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Mycorrhizae: its importance in Indian context

For the last three decades or so developing countries have been experiencing a continuous process of destruction and degradation of tropical forests.

New forest types can be established in degraded areas with the help of mycorrhizal technology which involves very negligible expenses and little management requirements. The importance of mycorrhizal association in maintaining the stability of fragile tropical forests is well recogniged.

Plants introduced to eroded sites or semi-arid, arid lands face lot of adverse conditions such as high sterility, alkalinity, salinity, low fertility and drought (water stress). Mycorrhizal fungi are known to subside these effects on plant growth. The soils in semi arid and arid zones are deficient in water and minerals particularly phosphorus. The vesicular arbuscular mycorrhizal (VAM) fungi holds immense importance in aforestation of waste and arid lands. These fungi help in better establishment of seedlings by taking up phosphorus and water with the help of extended root system.

Mycorrhizal plants produce significantly more dry matter than non-mycorrhizal controls and it also promotes the height of the plants. Though extent of mycorrhizal association in tropical forest species is well documented, information on its interaction in ecosystem function is less understood.

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Isolation of VAM Fungi - Lab Work

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Numberous techniques are used to recover VAM propagules from soil. The most basic of these is wet sieving and decanting to remove the clay and sand fractions of the soil while retaining spores and other similar-sized soil and organic matter particles on sieving of various sizes. This technique is relatively fast but further purification of the spores is usually necessary particularly if spore numbers in a soil are low. In addition, dense spores or sporocarps may be lost along with the sand fraction.

<u>Wet sieving and Decanting techniques</u>.

1. Mix a volume of soil (250 cc) in water (1000 ml) and allow heavier particles to settle for a few seconds.
   Note: If the number of spores contained in various soils is to be compared, the volume of soil sieved should be correlated to the dry weight of that soil sample.

2. Pour liquid through a coarse soil sieve (500-800 m) to remove large pieces of organic matter. Collect the liquid which passes through this sieve. Wash the sieve in a stream of water to ensure that all small particles have passed through.

3. Resuspend the particles in the liquid which passed through the coarse sieve and allow heavier particles to settle for a few seconds.

4. Pass this suspension through a sieve fine enough to retain the desired spores, generally 38-250 m (If total population is to be assessed, the finest available sieve should be employed).

5. Wash the material retained on the sieve to ensure that all colloidal material passes through the sieve.

6. Transfer small amounts of the remaining debris to a petri dish and examine under a dissecting microscope.

Note: A nest of varied-sized sieves may be used instead of steps 2, 3, and 4. If a nest of sieves is employed, care must be taken to ensure that the finest sieve does not overflow, losing siev ings. If the bottom sieve does 'clog up' either a strong stream of water or patting the side of the sieve will often unclog it.

Gerdemann, J.W. and Nicolson, T.H., 1963. Spores of mycorrhizal Endogone extracted from soil by wet sieving and decanting. Trans. Br.Mycol. Soc.46: 235-244.

VA MYCORRHIZAL INOCULUM PRODUCTION

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Interest in VA mycorrhizal fungi has reached a pinnacle by the research community. The ability of these fungi to produce a dramatic response in plant growth is well documented. However, the application of this technology to commercial production of food, fiber or fuel has been minimal. This is mainly because of the difficulties in inoculum production, the fungi being obligate symbionts. Attempts to culture VAM fungi on artificial media has met with little or no success. At the present time, the only method to produce these fungi is in association with the host plant root. Novel techniques to produce VAM inoculum in almost sterile environment through nutrient film technique or circulatory hydroponic culture system have not reached commercialization because of many limitations. So large scale production of VAM inoculum is technically feasible using traditional 'pot culture' technique developed about 30 years back by Barbara Mosse and Jim Gerdemann. However, the different methods used for production of VAM inocula are briefly discussed.

1. Spores of VAM fungi as inoculum

This may be a suitable method for experiments and for special cases, ex: plants grown invitro. Nopamornbodi et al. (1987) applied two hundred VAM spores in water suspension under each seed of soybean and obtained increased yield undef field conditions. Large scale production of spores is difficult. Considering 100 spores/g of inocula , 200-2000 kg inocula may have to be sieved to get desired number of spores (about 100 spores/seeding point) for inoculating one ha of field sown crop. It may be feasible for nursery raised crops. Because of the existence of spore dormancy, spores cannot germinate and compete effectively with native endophytes which can infect host from hyphal bits and infected root bits.

2. Infected roots as inoculum source:

Large scale production of infected roots is possible in aeroponic cultures (Sylvia and Hubbell, 1986). Infected roots contain internal mycelium and external mycelium (may have spores). Infected roots can colonize the host after one to two days of inoculation. Root inoculum without spores should be used within a week. In vitro reproduction of VAM fungi on tissue cultured roots has been demonstrated (Nopamornbodi et al., 1988). The production process is difficult and expensive. Other problems are: a. Infected root introduced as inoculum acts as an attractive nutrient source for several saprophytic and parasitic microorganisms. b. Short survival time and C. large quantities of the inoculum required. On the basis of 10 g of root inoculum per cassava plant, about 100 to 150 kg root inoculum would be needed per hectare (Howeler et al., 1987). For beans 400 to 800 kg/ha of root inoculum could be required on the basis of 2 g of root inoculum per seed (Sieverding and Saif, 1984).

3. Soil based inoculum

Soil inoculum produced using a traditional "pot-culture" technique contains all VAM fungal structures and is highly infective. The success of good soil inoculum production depends on the selection of host-plant and the ambient conditions under which a defined VAM fungus can be mass multiplied. Recently, we conducted a series of experiments and found that Rhodes grass as the best host, perlite-soilrite mix as the best substrate and calcium ammonium nitrate and rock phosphate to be the best N and P sources for mass producing Glomus fasciculatum (Sreenivasa and Bagyaraj, 1990).

4. Inoculum production in peat (Nutrient Film Technique)

Warner et al. (1985) used sphagnum moss peat mixed with three times its weight of water and lime to obtain the pH suited for the mycorrhizal fungus. VAM inocula obtained from pot cultures are incorporated into the peat and compressed into blocks of 4X4X4 cm. Lettuce is allowed to grow in the peat block for 2 to 5 weeks. Then the blocks are transferred to nutrient film technique (NFT) channels (Cooper, 1985). The NFT channels slope and the nutrient solution flows at 200 ml/min. Plants are allowed to grow in NFT system for 8 to 10 weeks. During this time, mass reproduction of the fungus

takes place. The peat blocks are allowed to dry, chopped and used as VAM inoculum. The shelf-life of this preparation is around 6 months. During the production process in moist peat contamination with root-pathogens is possible.

5. Expanded clay as carrier of VAM fungi.

Light expanded clay aggregates have been used as a carrier for mass multiplying VAM fungi in Germany (Dehne and Backhaus, 1986). VAM fungi sporulate within the porous inorganic particles. Clay granules of 4 to 10 mm are optimum for the reproduction of VAM fungi. In expanded clay, the inoculum can be produced in a relatively short time in large greenhouse. The carrier being an inorganic material, there is less contamination by pathogenic organisms. The inoculum is light and hence easy to transport. The shelf-life of the preparation is upto 5 years. The product is granular and hence, can be used with agricultural machinery.

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VA Mycorrhizal Dependency of Plants

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Plants differ greatly in their need for and response to mycorrhizal infection. Gerdemann (1975) defined relative mycorrhizal dependency (RMD) as the degree to which a plant is dependent on the mycorrhizal condition to produce its maximum growth or yield at a given level of soil fertility. This was based on determining the extent of growth increase attributed to the mycorrhizal condition. Menge et al (1978) proposed the following formula to calculate mycorrhizal dependency of crop plants using an inoculant vesicular-arbuscular mycorrhizal fungus in sterilized soil.

$$RMD = \frac{\text{Dry weight of mycorrhizal plant}}{\text{Dry weight of non-mycorrhizal plant}} \times 100$$

The drawback of this formula is that RMD values go beyond 100% making it difficult to categorize crop plants.

Plenchette et al. (1983) proposed another formula to calculate relative field mycorrhizal dependency (RFMD) of crop plants under field conditions. They compared plants in fumigated and non fumigated soils. This measure the extent of growth increase due to native endophytes. The calculated values lie between 0 and 100%.

$$RFMD = \frac{\text{Dry weight of mycorrhizal plants} - \text{Dry weight of non-mycorrhizal plant}}{\text{Dry weight of non-mycorrhizal plant}} \times 100$$

This formula is of great practical importance for ascertaining the response of crop plants to native mycorrhiza in fields before applying field inoculation to crops.

There have been many reports that the introduction of mycorrhizal fungi can improve plant growth under unsterile soil conditions in spite of the presence of native endophyte. Hence, Bagyaraj et al. (1988) proposed another formula which enables calculation of the mycorrhizal inoculation effect (MIE) to assess the growth improvement brought about by inoculation with a mycorrhizal fungus in unsterile soil with indigenous vesicular-arbuscular mycorrhizal fungi.

$$\text{MIE} = \frac{\text{Dry weight of inoculated plant} - \text{Dry weight of uninoculated plant}}{\text{Dry weight of inoculated plant}} \times 100$$

MIE is very useful for the assessment of the extent to which introduced fungi compete with native endophytes to bring about plant growth response. This information is of great value in practical agriculture, especially in developing countries, where farmers do not fumigate or sterilize either nursery or field sites.

Factors affecting VAM dependency: The extent to which a plant species is dependent on VAM fungi to produce its maximum growth or yield is significantly influenced by soil, plant and fungal factors. Soil factors seem to be more important. Among key soil factors that are believed to regulate the effectiveness of VAM symbiosis, P has been extensively investigated. Hayman (1986) noted that it is difficult to grade the responsiveness of plant species to VAM colonization based on reports in the literature. This is because the vast majority of the investigations were based on the amount of soluble P based rather than on the concentration of soil solution P, which makes soil-to-soil comparisons impossible. Such investigations may have to be conducted by establishing P levels in the soil solution using P absorption isotherms (Habte and Manjunath, 1987), which may permit soil-to-soil comparisons since external P requirements of crops are known to be relatively constant under a wider range of soil conditions (Fox, 1981) Such an investigation involving 9 levels of the soil solution indicate the existence of threshold, optimum and inhibitory levels of P for the symbiosis (Habte and Manjunath, 1987).

Therefore, it appears that predicting mycorrhizal dependency of a plant species snould be determined at the soil solution P concentration determined to be optimal for the VAM symbiosis.

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IDENTIFICATION OF VAM FUNGI

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"A science can scarcely be said to exist before its material, whether it be species or elements or geologic and geographic features, has been organized and classified" (Wertenbaker, 1974). VA mycorrhizal fungi are no exception, they require description, names and keys to taxonomic of not biological species to allow some coherent understanding of the biological and ecological role of mycorrhiza in forest ecosystems. The taxonomy has been largely based on morphological data and also through the use of biochemical and molecular data. Taxonomy may not revolutionize theories on mycorrhiza but can clarify some of the controversy in the literature which has arisen as a result of misrepresentation.

Vesicular arbuscular mycorrhizal fungi are ancient organisms. Fossil plant roots dating to the palaeozoic era have been observed with structures resembling those produced by extant VA mycorrhizal fungi. Much speculation prevailed about these fungi until they were cultured on the roots of plants in pots by Mosse (1953) and their propagules were isolated by wet sieving them from soil. This enabled researchers to study these fungi experimentally for the first time, removed from their natural habitat. They are now known to be obligate symbionts belonging to zygomycotina culturing these fungi on culture media has failed. Only possibility remains is raising the soil-root bit inoculum by pot culturing of vesicular-arbuscular mycorrhizal fungi. Inoculate surface sterilized spores of known VAM fungi with host plant growing in sterilized sand or alfisol soil. Hoagland nutrient solution minus phosphorus is used as nutrient solution. Collect the inoculum after 2-3 months either in the form of spores, extra matrical mycelium

or soil. Host plant roots can also be used as inoculum. Rhodes grass, maize, sorghum, Cenchrus ciliaris, legumes and castor can be used as suitable host plants for multiplication in the mass inoculum production.

VAM fungi are ubiquitous in occurrence and distributed throughout the world. They occur in the rhizosphere soil and colonize roots of cultivated crops, grasses, shrubs, herbs and majority of forest tree species. It is also established that 95% of plants are colonized by VAM fungi and plants can not grow adequately without VAM fungi, especially in phosphate deficient soils.

Taxonomic Studies

The most interesting, important, difficult and fundamental problem is the identification of VAM fungi. It relies mostly on spore morphology, nature of sporocarp size, septa, hyphal attachment, colour, extra matrical hyphae or structures, ornamentation, presence or absence of germination shield and wall layers VAM fungi are formed by certain species of Endogonace family under Endogonales of zygomycotina. Sporocarps are also known to be present which contain azygospores or chlamydospores and sporocarps vary in size.

The taxonomy of the group presents difficulties, because early workers failed to appreciate the distinction between zygosporic, azygosporic and chlamydosporic forms. However Gerdemann and Trappe (1974) have separated seven genera. The genus Endogone is restricted to forms with true zygospores. However, it is not known to produce vesicles and arbuscules, hence cannot be considered as typical VAM fungus. In another genus such as Glaziella, the sporocarps are hollow.

A sexual reproduction by means of sporangia is uncommon in VAM fungi, but is found in Modicella. The true nature of VAM fungi was not exhibited by Glaziella and Modicella, hence not considered. Acaulospora Entrophosphora, Gigaspora, Glomus, Sclerocystis and Scutellospora are the valid genera of VAM fungi.

Morphology

The hyphae of VA mycorrhizal fungi develop mycelia, arbuscules, and/or vesicles in the root. In addition, extra matrical hyphae grow from the root out into the rhizosphere soil. The hyphae are largely non-septate and can extend several centimeters beyond the root surface. Thus these hyphae can explore an area around the root which far exceeds that available to root hairs. It is the ability of these hyphae to absorb relatively immobile elements in these additional areas in soils that enables these fungi to benefit plants, especially those with coarse root systems.

The spores which are produced by VA mycorrhizal fungi are largely borne on the extra matrical hyphae beyond the root surface. However, 10 of the 126 described species of these fungi also produce spores within the root. The extra matrical spores can occur singly in the soil, in loose clusters of spores on hyphae or in tightly packed masses with or without a covering of hyphae enveloping them. The manner in which the spores are borne and the manner in which they arise on the hyphae are the criteria used to separate the genera of these fungi. There are keys which may be used to separate these species (Hall, 1984; Trappe, 1982) and a manual containing the

descriptions of all recognised species (Schenck and Perez, 1988). In addition an excellent review of the criteria used to speciate these fungi (Morton, 1988) is also available. However, speciation of VA mycorrhizal fungi is not easily accomplished.

VAM fungi have an extensive loose hyphal network that extends a considerable distance into the soil. The hyphae also grow along the root surface and tightly appressed to the epidermis. The external hyphae are dimorphic and are composed of coarse, thick walled, irregular, non-septate hyphae with smaller thin walled internal branches.

The internal morphology of VAM is readily observed by cleaning and staining technique (Phillips and Hayman, 1970). The chlamydospores germinate in close proximity to roots, and develop germ tubes which penetrate the epidermal cells. Soon after infection the fungus forms branched haustoria like structures called arbuscules. At intervals with in the root tissue, either inside or between the cells, large terminal or intercalary thick walled vesicles may develop. These are multi-nucleate and their size and shape depend on the host and nutrient conditions. When young they have thin walls and contain a homogeneous protoplasm. They may remain thin walled and function as storage organs for food or develop into thick walled chlamydospores functioning as reproductive structures.

Fungal mycelium and its parts

Some of the reported diagnostic hyphal characters that help to seggregate genera are :

1. Long infection units with 'H' connections between parallel strands

   - Glomus

2. Pale straining by trypan blue and constrictions near branch points

   - Acaulospora, Entrophospora

3. Coiled, irregularly swollen hyphae with lateral projections or knobs

   - Gigaspora, Scutellospora

Morton (1990) uses arbuscules as the only character to unite Glomineae and Gigasporineae in Glomales.

1. Abrupt narrowing of branch hyphae

   - Gigaspora

2. Incremental reduction in hyphal width

   - Glomus

Vesicles of Glomaceae are subglobose to elliptical where as in Acaulosporineae they are pleiomorphic, knobby, stain lightly. Auxillary cells are found only in genera of Gigasporineae.

The shape and length of cell wall ornamentations differ enough to separate genera, but a continuous of variation in each genus makes

these characters taxonomically uninformative at the species level.

Spores and sub-cellular structures

1, Spores are formed on cylindrical to flared sporogenous hyphae

- Glomus

2. Compact sporocarps

- Sclerocystis

3. A saccule forms terminally on a sporogenous hypha, after which spores are initiated laterally from or within this hypha

- Acaulospora, Entrophosphora

4. Spores expand from a bulbous sporigenous cell

- Gigaspora, Scutellospora

Spore wall

1. Spore wall consists of rigid laminae with one or more outer layers that slough (evanscent)

- Glomus, Acaulospora

2. Spore wall alternatively consists of laminae with permanent outer layer

- Gigaspora, Scutellospora

Molecular characters

Molecular characters include base sequences or restriction fragment, length polymorphisms of DNA regions, Isoenzymes, Glycoproteins, fatty

acids etc. Proteins and glycoproteins are identified by immunological procedures. Iso- enzymes have been used to extract information on taxonomic relationship in glomales. Fatty acid analysis has not been applied extensively.

At the present time, taxonomic information is not complete enough for even the most experienced individuals to make accurate identifications on all isolates they encounter.

The following steps are suggested :

1. Provide a culture of the experimental organism alone or with other members of native population to a living culture collection such as INVAM so that it can be studied by others.

2. Worry less about species identification than careful characterization of the spore phenotype to ensure that organism used for experiment is the same one existing at the end.

3. Prepare vouchers of spores and deposit them in a safe place so that identification may be determined or verified at some time eg. herbaria - INVAM.

4. Substitute an isolate name in place of the specific epithet when identifications are impossible or difficult.

Any thing is better than educated guesses that mislead rather than inform.

Key for separation of VAM fungal genera

1. a) Spores produced as Chlamydospores-2

   b) Spores not produced as Chlamydospores-3

2. a) Spores only [illegible] radiating from a central core of hyphae in the compact sporocarp

   \- <u>Sclerocystis</u>

   b) Spores formed singly in soil or in sporocarps, if in sporocarps spores not radiating from a central core of hyphae

   \- <u>Glomus</u>

3. a) Azygospores formed near or below a swollen hyphal terminus-4

   b) Azygospores formed on a swollen hyphal terminus-5

4. a) Spore formed laterally on hypha, below a swollen hyphal terminus

   \- <u>Acaulospora</u>

   b) Spore formed with in the hypha, below a swollen hyphal terminus

   \- <u>Entrophospora</u>

5. a) Spore with 2 or more wall groups, the inner containing a coriaceous or membranous wall

   \- <u>Scutellospora</u>

   b) Spore of only one wall group, auxiliary cells echinulate or finely papillate

   \- <u>Gigaspora</u>

Monographs for VAM fungi identification

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within a flexible inner wall. Thin-walled, knobby or broadly papillate auxillary cells borne in soil, on straight or coiled hyphae, formed singly or in clusters.

The six genera that contain about 120 species, known to produce VA mycorrhizae with plants, are listed here. One must remember that VA mycorrhizal fungi are entities that do not always conform to the characteristics described in the original description. Some slight changes may be observed in at least size, shape, colour and perhaps other characters as well. Slight variation from the original species description would most probably be due to natural variations in a population. However, discovering a new taxon is also a possibility.

SPECIES DESCRIPTION

The six genera of Endogonaceae that contain about 120 species, known to produce VA mycorrhizae with plants, are listed here. One must remember that VA mycorrhizal fungi are entities that do not always conform to the characteristics described in the original description. Some slight changes may be observed in at least size, shape, colour and perhaps other characters as well. Slight variation from the original species description would most probably be due to natural variations in a population. However, discovering a new taxon is also a possibility.

Species Guide

Acaulospora	Spore size (µm)	No. of wall(s) (including layers)	Width (µm)
appendicula	(170-) 250(-390)	4	16-40
bireticulata	150-155	3	3.0
delicata	80-125(-150) X80-110(-140)	4	4.8-6
dilatata	(78-)106(-130)	5	9-13
elegans	140-285 X 145-330	3	≤16
foveata	185-310(-410)X215-350(-480)	3	14-18
gerdemannii	200-250	2	2-3
lacunosa	(98-)131(-186);98-147X131-182	5	4.6-13.2
laevis	119-520	3	2-4
longula	(55-)70-90(-100);100-115X 66-98	5	2.5-5
mellea	(72-)95-105(-126);96-130X 78-92	5	4-8.5
morrowae	(63-)79-92(-120);86-100X64-96	5	3.5-5.5
myriocarpa	(22-)32-90;(23-)28-95(-114)X 21-)28-80(-96)	3	1.5-3.5 (-7)
nicolsonii	99-198 x 109-218	2	5-13
rugosa	(49-)92(-118)	5	4-8
scrobiculata	100-240 X 100-220	4	4-8.5
spinosa	100-298 X 100-335	3	4-12
sporocarpia	(140-)160-200(-240)X(125-) 150-175(-200)	1	9-20
trappei	42-99 X 42-70	1	1.2-4
tuberculata	255-340	3	10-16.5

[illegible]			
colombiana	(75-)100-115(-135)	5	3-7
infrequens	69-183(-225)X69-164	2	6-14
schenckii	(37-)50-60(-77);48-97X25-60	3	1.5-2.5

[illegible]	Suspensor	spore size µm	No. of walls	width µm
[illegible]	[illegible]-36(-50)	143-330(-350); 230-252X234-250	1-6	4-12
[illegible]	[illegible]	200-300	2	<7
[illegible]	<65	320-490	3	20-47
[illegible]	[illegible]	183-500 X 291-812	2	2.5-7.5
[illegible]	[illegible]	260-[illegible]	1	5-24
[illegible]	[illegible]-40	230-305	2-5	2.4-7.5

Glomus	Spore size µm	No. of walls	Width µm
aggregatum	(20-)40-85(-120);(30-)40-120(-210) X (20-)40-85(120-)	1-2	2-6(-10)
albidum	(85-)95-168(-198)X(85-)95-168(-177)	2	1-4
ambisporum (formed singly)	54-197X44-163	2-3	6-9
formed in sporocarp	85-157;98-166X93-157	3	5-18
australe	(120-)160(-180)	2	11-19
boreale	100-145	1	8
botryoides	145-250	2	5-7
[illegible]	[illegible]-279 X 120-272	2	6-10(-16)
canadense	54-80(-100)	1	4
citricolum	35-65 X 60-90	2	4-10

claroideum	(70-)130(-180);59-126X72-145	1-2	(4.5-7.6(-10)
clarum	(68-)190(-290)	2-5	7-31
constrictum	150-330	1-2	7-12(-15)
convolutum	51-193X67-193	1	8-15
delhiense	100-125	2	10-12
deserticola	(47-)54-115X(38-)52-102	1	1.5)2-2.5(-4)
diaphanum	(39-)74(-121);36-86X64-132	2	2.2-7.8
dimorphicum	90-300 (single) 50-130 (clustered)	3 2	5-17 3-6
etunicatum	68-144(162)	2	4-13
fasciculatum	35-105; 75-150X35-100	1	3-17
fecundisporum	(60-)107(-155);83-150X107-207	2	1.5-7.5 (-13)
flavisporum	149-230 X 95-152	1	10-13
formosanum	65-135	1	5.5-12.5
fragile	53-73X49-62	2	2.5-3.5
feugianum	65-80	2	
fulvum	45-115	1	2-4
geosporum	110-290 X 100-290	3	5-18
gerdemannii	140-198 X 149-230	5	5-10(-13)
globiferum	150-270	3-4	8.5-36
glomerulatum	40-70	2	4.5-9.5
halonatum	200-280	2	18-35
heterosporum (formed singly)	99-206 X 61-201	3	[illegible]-10
in sporocarp)	31-102 X 27-68	[illegible]	3-4.5
hoi	(50-)80-120(-155)X(45-)75-120(-140)	[illegible]	([illegible]-)5-[illegible]
intraradices	(40.5-)98.5:(-190.5)93-119X112-131	1-4	3-1[illegible]
invermaium	50-75	[illegible]	

	(48-)175(-262);104-233X155-262	1	1.5-7.4(-10.6)
macrocarpum	(90-)120(-140)X(70-)110(-130)	2	7-14
maculosum	(95-)130-187(-220)	3	4.8-15
magnicaule	125-175	2	9-24
manihotene	(145-)170-235(-450);125-236	2 or more	(7-)10-16 (-40)
melanosporum	166-277X129-244	1	8-13
microaggregatum	(15-)30(-50)X(15-)30(-40)	1-2	<1-2.4(-4)
microcarpum	(30-)40(-45)X(30-)35(-40)	1	<7.0
monosporum	140-330	2	4-10
mosseae	105-310 X 110-305	2	2-7
multicaule	149-249 X 124-162	1	8.6-34
multisubstensum	100-150	2	10-15
occultum	15-100 X 20-120	1-2	(2-) 2.5-3.5(-6)
pallidum	32-78 X 28-68	1	1-8
pansihalos	(108-)130-155(-200)X(110-)120-130(-180)	3	7-15(-55)
pubescens	20-48 X 18-45	1	3-6
pulvinatum	53-90 X 55-93	2	2-5
pustulatum	(43-)86-140X(60-)80-140	3	<5-10(-23)
radiatum	60-110(-120)X48-75(-90)	1	4-8
reticulatum	130-170	2	10-15
scintillans	180-210	3	7-10
segmentatum	52-100 X 40-86	2	4-8
tenebrosum	(200-)240(-270)X(205-)230(-270)	1	(13-)18(-26)
tenerum	44-130	2	<8
tenue	10-12	1	<2.5
tortuosum	(120-)160(-210);94-180X112-230	1	0.5-2.0
tubaeforme	23-35	1	3-4(-15)
versiforme	(125-)160(-180)X(120-)140 (-175)	2	6.5-12
vesiculiferum	49-85(-100)	?	4-8

clavispora	460-750X590-780	140-185X(20-)25-40 (-50)	1	1.5-5-17-25
coccogena	400-675	100 X 40-50	-	--
coremioides	340-600	50-86(-102)X35-52 (-82)	1	2-4
dussii	263-540	50-80 X 32-54	1	2-3
microcarpus	100-420	95-115 X 40-60	1	3.5-17-26
pachycaulis	170-230X175-270	27.5-60 X 37.5-87.5	2	1-5
pakistanica	520-590(-700)	85-205 X 33-55	1	2-4
rubiformis	180X180-375X675	37-125X29-86	1	3-7.6
sinuosa	248-412	45-118X30-83	1	1.3-5.0

Scutellospora	Suspensor(μm)	Spore Size μm	No. of walls	With (μm)
alborosea	21-50	204-287;345X267	3	6.2-18.6
aurigloba	40-70	200-420(-520) X130-420(-520)	2-4	7-17
calospora	33-48	114-285(-511)X 110-412(-511)	4	5-9
coralloidea	55-64	308-393X294-454	3	9-17
dipapillosa	27-40	135-160X135-180	5	7.7-17.5
erythropa	30-60-125X30-60	(170-)221-364(-551)X 205-314(-660)	4-5	5-19
fulgida	26-46	160-240X160-245	3	7-9.5
gilmorei	27-40	204-320	6	18.4
gregaria	39-80	250-448X250-480	4	10-23
heterogama	21-42	150-253	4	6.5-12
minuta	22-31	97-180	3	6.5-9
nigra	40-60X80-120	297-500(-1050)	2	7-10
pellucida	10-29(250)	58-183(-250)X58-241 (-410)	6	6-18
persica	47-62X47-125	270-354X281-384	3	3-12

reticulata	45-87X84-140	208-470X188-340	6	9.8-17.3
savannicola	33-51	288-581X214-364	5	9.1
tricalypta	14-47	303-387;456X257	3	17
verrucosa	55-100X35-70	220-476X220-476	3	6-13
weresubiae	32-50	125-265X135-414	6	8-20

Acaulospora appendicula Spain, Sieverding & Schenck

Azygospores formed singly in the soil adjacent to roots; borne on a short, hyphal pedunculate protuberance, 30-100 µm long, 20-50 µm wide, arising from a tapering hyphal terminating in a globose, swollen hypha terminus. (190-)250(-380) µm diam. with a wall 1-6 µm which consisting of an outer wall of overlapping plate-like structures and a membranous inner wall; hyphal terminus contents white-opaque when newly formed; hyphal terminus becoming grey-white, subhyaline, or transparent when contents empty to form a spore; hyphal terminus usually persisting on young spores, generally not collapsing when detached from the spore at the juncture of the hypha and the pedicel. Azygospores (170-)250(-390) µm diam, white-opaque when young, becoming dull yellow-cream to orange-tan when mature; hyphal pedunculate protuberance attached to the reticulate inner wall and forming an appendage on the spore; azygospores with two separable walls each consisting of two walls not readily separating; the outer wall 8-16(-20) µm thick somewhat roughened, becoming yellow to brown with age, with an irregular, reticulate pattern of fine cracks that serve as fracture lines when an azygospore is crushed; second wall hyaline, 2-6 µm thick, with and alveolate reticulum: reticulum 8-12 X 4-8 µm: third wall hyaline;

SAMPLE PREPARATION FOR IDENTIFICATION OF VAM FUNGI

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Considerable interest in VA mycorrhizal fungi has been expressed by researchers throughout the world. These fungi have a greater role in 'P' uptake and benefit the plants. Plants with a good compliment of VAM fungi can have reduced plant stress in saline soils, from plant pathogens, from drought, from transplant injury and from heavy metals in soil. The presence of VAM fungi in the roots of plants increased the production of phytohormones and growth regulators. The mycelia of these fungi stabilise soils. These fungi also seem to be very selective in their occurrence and all of them do not fill the same ecological niche.

Procedures for isolation of VAM fungi

Most VA mycorrhizal fungi sporulate outside the plant root in the soil. However there are 10 species of Glomus which readily sporulate in the root. Even spores of many of these species also occur in soil, since isolates vary in their ability to sporulate inside and outside the root. Therefore it is logical that for isolation of these fungi one would use a process that was capable of removing propagules from soil. Gerdemann (1955) was first successful at this using a wet sieving process that nematologists commonly used to extract nematodes from soil. Since VAM fungi are obligate symbionts, they could not be isolated from soil using dilution plate methods, selective media and other procedures.

Modified wet sieving and decanting procedure (Gerdemann and Nicolson, 1963) is the commonest procedure and the details are as follows :

1. Add 50 to 250 g soil to one litre of tap water in a beaker.

2. Break up the large soil clumps by hand and stir the mixture for 10-15 seconds.

3. Allow the coarse particles to settle in the water for 5-10 seconds.

4. Decant the soil-water mixture through stacked sieves with the coarse sieve on top and the fine sieve on the bottom, the range of coarse sieve openings should be 420-250 µm, for sieves with medium size openings, and 105 to 45 µm for the sieves with fine pore openings.

5. Steps 1-4 should be repeated atleast once to increase the likelihood that a majority of the spores in the soil have been removed.

6. The contents on each sieve should be back washed onto a 15 cm petriplate so that the enough material is added to cover the dish bottom with a single layer of detritus. More than this amount will obscure spores and make them difficult to see or remove.

   <u>or</u>

   The collected debris of each sieve was filtered separately through Whatman No. 1 Filter paper or single synthetic fibered imported white cloth. The filter paper/cloth may be kept in a petri-dish with some water.

7. Examine the contents for spores or sporocarps under a dissecting microscope under a reflected (20-100 x) light glass micropipetts, microspatulas etc can be used.

The various steps after examination are

1. Preparation of a 2 sided diagnostic slides with spores. Polyvinyl alcohol - lactophenol be used as mountant. Both broken and unbroken spores be observed.

2. Detailed examination of spores with compound microscope (100 - 1000X). Use of phase contrast may be of great help in determining wall layer characteristics.

3. Recording the data, measurements etc on to the INVAM worksheet ocular and stage micrometers be used in micrometry.

4. Determination of the genus may be made using keys provided in standard manuals.

5. Identification of species

6. Comparison with original description from a standard manual or from related species.

7. Select the most appropriate species based on comparison.

References

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Morton J B 1986 Effects of mountants and fixatives on wall structure and Melzer's reaction in spores of two Acaulospora species & Endogonaceae) Mycologia **78** : 787-794.

Trappe T M and N C Schenck 1982 Taxonomy of the fungi forming endomycorrhizae. A vesicular arbuscular mycorrhizal fungi (Endogonaceae) Mycologia **78** : 787-794.

Schenck N C and Yvonne Perez 1988 Manual for the identification of VA mycorrhizal fungi 2nd Ed INVAM Univ. of Florida, Gainesville, USA.

David P Labeda 1990 Isolation of Biotechnological organisms from nature, McGraw Hill Publ. Co., N.Y., London, Sydney, Tokyo, Toronto 322 pp.

Note : Work sheet enclosed.

INVAM WORKSHEET

(See the Glossary after Chapter 6 for the definition of unfamiliar terms.)

YOUR NAME ______________________ DATE __________

SLIDE LABEL INFORMATION ______________________

SPECIMEN TENTATIVE IDENTIFICATION ______________________

I. Many of these observations can be made with a dissecting microscope but measurements should be made with a compound microscope.

A. Spore color:

1) In water __________

2) In mountant __________

Mountant used: PVL ______ PVLG ______ Other ______

B. Spore diameter: __________ __________

For globose spores: Range __________ µm Mean __________ µm

For irregularly-shaped spores:

1) Length: range __________ µm Mean __________ µm

2) Width: range __________ µm Mean __________ µm

C. Composite spore wall thickness (determined on intact spores if possible)

__________ µm

(See Part II for composite spore wall components measurements.)

D. Attachment present? Yes ____ No ____ If no, go to D.

1) Hyphal terminus (=sporiferous saccule) present? Yes ____ No ____

2) Bulbous suspensor (= sporogenous cell) present? Yes ____ No ____

3) Hypha(e) present? Yes ____ No ____

E. Spore contents:

1) Globular ______ 2) Reticulate ______ 3) Granular

H. Auxiliary cells present? Yes ____ No ____ If no, go to

If yes, check which applies:

1) Knobby ____ 2) Digitate ____ 3) Coralloid ____

4) Echinulate ____ 5) Spiny ____ 6) Pigmented ____

If pigmented, indicate color __________

I. Sporocarp present? Yes ____ No ____ If no, go to J.

1) Sporocarp diameter __________

2) Peridium present? Yes ____ No ____ If yes, indicat

J. Additional comments __________

K. Determine the genus of your specimen. (See Chapter 3.)

Genus ______________________ Go to I

1) For *Gigaspora* or *Scutellospora*:

a. Bulbous suspensor (=sporogenous cell) dimensions:

Width ____ µm length ____ µm

b. Subtending hyphae (=sporophore hyphae) septate? Yes

c. Surface ornamentation on spore present? Yes ____

Description __________

d. Germination shield present? Yes ____ No ____

2) For *Acaulospora* or *Entrophospora*:

a. Hyphal terminus (=sporiferous saccule) present?

Yes ____ No ____ If no, go to II.

b. Hyphal terminus collapsed? Yes ____ No ____ If yes,

c. Terminus dimensions (diameter or length & width) ____

d. Description of terminus contents (color; content a granular, reticulate, globular; texture, e.g., smo

a. Pore occluded? Yes ____ No ____

b. Pore diameter ______________ µm

c. Presence of a septum at the pore? Yes ____ No ____ If no, go to d.

Protruding septum? Yes ____ No ____

d. Hyphal width adjacent to spore wall ____________ µm

e. Number of attachments per spore ____________

f. Outer wall of hypha contiguous with outer wall of spore?

Yes ________ No ________

g. Type of attachment: (Check all that apply)

(1) straight ____ (2) recurved ____ (3) funnel-shaped ____

(4) branched ____ (5) septate ____ (6) constricted ____

(7) swollen ____ (8) other: ______________

II. Make these observations on broken spores with a compound microscope. (See Chapter 4.)

A. Number of wall groups in the spore wall ____________

B. Width of each wall group:

A = ____ µm B = ____ µm C = ____ µm D = ____ µm E = ____ µm

C. Number of walls within each group:

A = ______ B = ______ C = ______ D = ______ E = ______

D. Type of wall(s) within each group: (See Chapter 4)

A = Amorphous
C = Coriaceous
E = Evanescent (ephemeral)
L = Laminate
M = Membranous
P = Hyphal peridium
U = Unit
X = Expanding

Wall Group A =

B =

C =

D =

E =

Walls ornamented or beaded are indicated by a subscript o or b, respectively, under the wall type.

Walls difficult to see are shown by a superscript over the wall type.

E. Wall reaction to Melzer's reagent. Indicate positive (+) or negative (-) for each wall group. If positive, also indicate wall color and wall type affected.

If other reagents or stains are used, record the spore wall reaction as for Melzer's reagent.

A ______________________________

B ______________________________

C ______________________________

D ______________________________

E ______________________________

F. Using the information from Section II, construct a murograph. (See Chapter 4)

G. Additional comments: ______________________________

____

Collection and preservation of Agaric

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Agarics can be found growing in plenty during the rainy season in living and dead trunks of trees and logs of wood, in the soil, in lawns, meadows, on dung and on termite mounds. But they develop and decompose very quickly in our tropical climate. This article deals with the methods of collection and preservation of agarics for scientific study.

The equipment needed for collecting mushrooms is not elaborate. A market basket with handle is essential. Baskets with lid may be useful when one gets caught in sudden rain during collection. In addition to the basket one should be equipped with sheets or newspapers, small tin cans or plastic boxes, a pen knife or a hunting knife, pencil and a pad of papers.

While collecting agarics it is always preferable to collect many specimens showing different stages of development of the fruit body. But this may not be possible always. If only a single good mature specimen is available, it can also be collected. Care should be taken while removing the fruit bodies. The specimens should carefully dug up from the substrate, along with the underground parts like valva, pseudorhiza etc., with the help of the knife and it should be handled as little as possible. Then the specimens should be placed on a sheet of newspaper in such a way that it can be wrapped once or twice and the ends twisted as if wrapping a sweet (Fig.1) After wrapping like this small specimens should be kept in tins or plastic boxes and the larger ones arranged in the basket. Each specimen should be given a number and notes should be taken about the associated plants, smell, change of colour when bruising etc. It is always preferable to collect specimens of about 10 different species during a single day's collection rather than collecting many species which cannot be worked out in a single day. It is ideal to start collecting as early as possible in the morning and devote the afternoon for studying the collections.

As soon as the specimens are taken in the laboratory a spore print of the specimen should be taken. For taking a spore print, the stipe of the specimen should be cut off and the cap placed on a piece of white card board or white paper, gills facing downwards. Use of black paper is not advisable because some of the lighter colours appear white in black background. The white spore deposits can be ascertained on a white paper by viewing the paper at an acute angle. Very small specimens may be placed on a clean slide with a drop of water over the cap to prevent drying up and preferably covered by a small container. Usually within an hour a good spore print can be obtained. While the spore print is getting ready one should proceed with taking notes of the macroscopical features of the specimen. Always start working with small, fragile and delicate specimens first. Though leathery forms can be studied later. Care should be taken to note all the characters which might change when the specimens are dried. For this a data sheet may be prepared so that one may not miss any character. A specimen copy of a data sheet which is used in our laboratory is appended at the end of this article. It is important to note the colour of the pileus and for this a standard colour chart should be used. There are many colour charts available in the market. The one which we use in our laboratory is *Metheun Hand-book of Colour*. It is not very expensive, easily available and many professional mycologists are now using it. A few sketches of the entire specimen and of a longitudinal section should be made in the data sheet. Care should be taken to show the exact position of the gill attachment to the stipe (Figs.2 & 3). When the spore print is ready the colour of the print should be noted with the help of the colour chart.

After the above mentioned studies are completed the specimens should be dried properly for future reference work. Drying is the only really satisfactory way of preservation. A simple drying apparatus can be constructed by any body. The two important principles to be kept in mind while making an apparatus are a steady source of heat and good circulation of hot air around and among the fruit bodies. A set of small screens (wire mesh) fixed on to a frame and arranged one above the other will serve the purpose. a hot plate can be used as the source of heat (Fig.4). If a hot plate is not available, a couple of electric bulbs (60 or 100 W) fixed to a wooden plank would be alright. The frame with the screens and the hot plate must be surrounded by a heat proof canvas. The lowest screen should be far enough from the heat source so that the wire of the screen becomes warm but not too hot to touch. The specimens should be kept directly on the screen and not over papers. When the specimens are dry they become brittle and if they are allowed to stand for 24 hours at room temperature they will absorb enough moisture to become plaint. In this condition they can be stored in cardboard boxes along with the labels. insect damage is one of the biggest problems in tropical climate. During drying the insects present on the specimens are completely killed. But after a short time small beatles find their way to the specimens and completely destroy them. To prevent this, a few crystals of paradichlorobenzene or similar insecticides should be placed in the boxes at regular intervals. This is very important.

Some books suggested for references :

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Smith, A H. 1973. The mushroom hunters' field guide revised and enlarged. The University of Michigan Press, Ann Arbor, 264 pp.

Zoberi, M H. 1972. Tropical macrofungi. Some common species. The Macmillan Press Ltd., London, 158 pp.

Specimen copy of Data Sheet

Name or Number : **Locality :**

Habitat : Soil type, Vegetation, on dung, vegetable debris etc.
Habit : - Solitary, in troops or rings
Collected by : Determined by :
Date :

Photo
Or
Drawing of exterior and vertical section of fruit body :

Pileus : Diameter Shape: Colour

Surface : dry, moist, viscid, glutinous etc.

smooth, scaly, shaggy, velvety etc.

Margin : regular, wavy Incurred or not
smooth, rough, furrowed striate or not

Veil, of present :
Colour
distribution at margin, whether appendiculate or dentate
filamentous or membranous

Gills remote, free, adnate, adnexed, decurrent etc.
crowded or distant
shape
thickness width
colour when immature at maturity
features of gill edge :

Stips : Central, eccentric or lacking shape
dimensions: length thickness
colour : when immature at maturity

Consistency (whether fleshy, stringy, cartilaginous, leathery or woody) surface characters (whether fibrillose, dry, viscid, scally or smooth)
veil, if present . Characters
valva, if present: Characters
ring if present :
Whether single or double Whether membranous or filamentous
Whether persistent, fugacious or mobile: Whether thick or thin
Whether apical, median or basal

Flesh : Colour in pileus When wet When dry
Colour in stipe When wet When dry
Colour change if any when exposed to air .
presence or absence of milk-like or coloured fluid.

Smell : before and after cutting : relate to a common odour

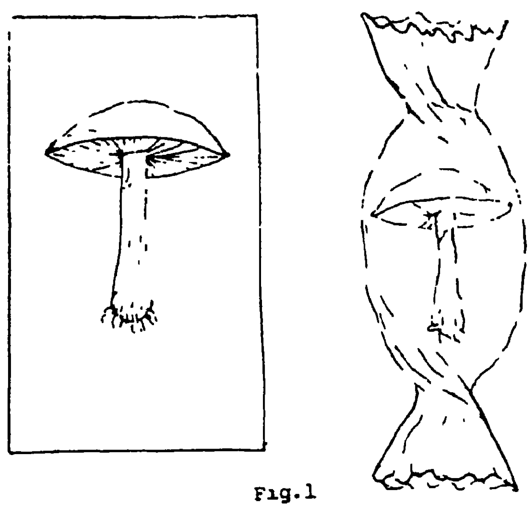


Fig.1

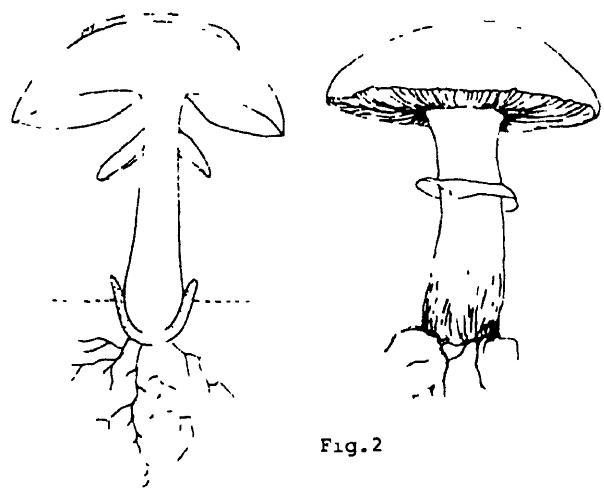


Fig.2

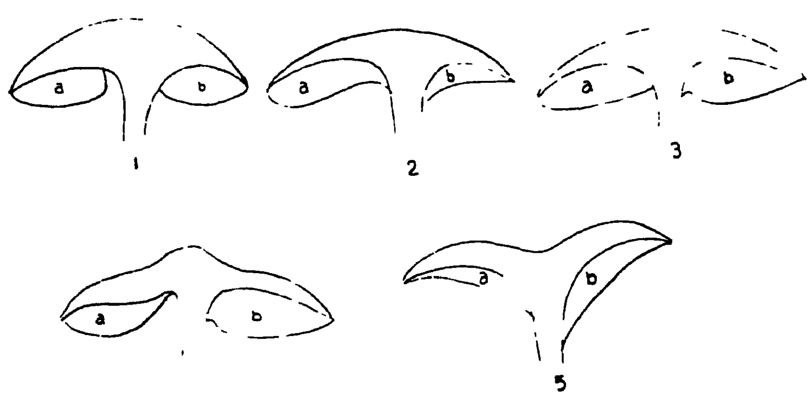


Fig.3

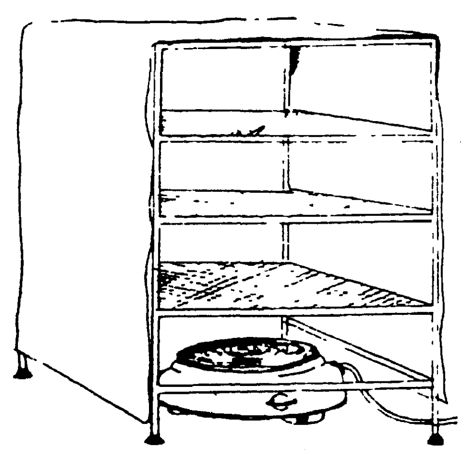


Fig.4

Fig.3. Different types of gill attachment:
1a. Remote, 1b. Free,
2a. Adnate with tooth
2b. Subdecurrent
3a. Adnate, 3b. Alucinate
4a. Adnexed, 4b. Emarginate
5a. Decurrent, 5b. Arcnate

Structure of Ectomycorrhizas

Collect ectomycorrhizal samples either directly from the trees in the plantations or from the seedlings grown in nursery bags in sterilized soils inoculated with specific fungi.

Soak the mycorrhizas in water and clean the debris with fine needles, forceps and brushes.

Select morphologically distinct mycorrhizal pieces based mainly on the colour of the mycorrhizal mantle and form of branching.

Take colour photographs of fresh mycorrhizas of different types under stereo microscope.

Place some fresh mycorrhizas of each type in sterile distilled water and store in refrigerator at 4^{o} C for studying the plan or surface views of the mantle layers and chemical reagent tests.

Preserve the other portions of the mycorrhizas in F.A.A. (13 ml formaldehyde solution + 200 ml ethyl alcohol + 5 ml glacial acetic acid) for the preparation of transeverse sections.

Characters used in the identification of ectomycorrhizas

Macroscopic features:

Examine the distinct types of mycorrhizas under dissecting microscope for the colour, forms, surface texture of the mantle, attached mycelium and attached rhizomorphs.

Colour of the mycorrhizas:

Colour is the most characteristic feature which should be recorded from fresh specimens. The colour terminology should be used from a standard colour chart such as Kornerup and Wanscher (1978).

Size and form of the mycorrhizas:

Record the length and breadth of the mycorrhizas and their forms (monopodial, bipodial or dichotomously branched, coralloid or clustered etc.).

Surface texture of the mantle:

Note the texture of the mantle surface (Smooth, cottony, wooly, velvetty, reticulate, granular, warty, fleshy or crusty).

Attached mycelium of the mycorrhizas:

Note the presence or abscence of the attached mycelium. Note also the microscopic features such as colour and origin (whether they radiate from the mantle surface or just seen on the surface) and microscopic features such as types of hyphae, their structure, size, presence or absence of wall deposits, clamp connections and hyphal branching.

Attached rhizomorphs of the mycorrhizas:

Record the presence or absence of rhizomorphs and its characteristic features. Note macroscopic features such as colour, abundance and morphology (flat, Sheet- like, thread like, feathery or wefty, free or directly connected with the mantle). Note also the microscopic features such as types of hyphae present, their structure, size, presence or absence of wall deposits, clamp connections and types of hyphal branching and nature of anastomosing.

Microscopic features:

Transverse sections of mycorrhizas

Take transverse sections (6-8 um thick) with the help of freezing microtome from the preserved materials of different mycorrhizal samples to study the anatomical details of mantle edge with emanating surface hyphae, cystidia, setae or bristles.

mantle thickness, tannin cells, cortical cells, Hartig net cells and the depth of the penetration of the Hartig net in the cortical cells.

Squashing and peeling techniques:

Study the plan or surface view details of the mantle by squashing and peeling technique.

Stain fresh mycorrhizas in cotton blue (0.1% cotton blue in 10% lactophenol / water) and toluidine blue (0.1% aqueous toluidine blue) separately on a clean microscopic slide and then firmly squash them under a cover glass.

Peel off small pieces of mantle with fine forceps and needles and then stain with cotton blue + toluidine blue. Distinguish mantle tissue into outer, middle and inner layers. Take photomicrographs of both transverse sections and plan view structural details of mantle tissues.

The line drawings of the different layers of the mantle as seen in the plan view can be redrawn from different photographic prints. This is done to give a more clear and detailed representaiton of the different mantle layers.

Descriptions of the mantle layers:

Plectenchyma

A thick tissue formed by hyphae becoming twisted and fixed together on which the individual hyphae are recognizable.

Prosenchyma

A type of plectenchyma consisting of hyphae of different length lacking individuality. It is a loosely organised structure in which the hyphal elements can be distingushed clearly with abundant inter hyphal spaces. This type is further subdivided into felt prosenchyma and net prosenchyma.

(1) Felt prosenchyma

It consists of sparingly branched hyphae with long 'cells' generally similar in appearance to individual hyphae of the same fungus in the soil.

(2) Net prosenchyma

It consists of shorter 'celled' hyphae branching frequently at wide angles. These hyphae are often slightly wider than individual hyphae of the same fungus in the soil and may bulge out a little between septa to produce a rather beaded appearance.

Synenchyma

It is a compacted tissue with little or few obvious inter hyphal spaces, in which the hyphal bases is difficult or impossible to discern (synenchyma = pseudoparenchyma).

Irregular synenchyma

It consists of wide hyphae subdivided into shorter cells and are probably frequently branched, but the degree of branching, the identity of the constituent hyphae to a large extent are immpossible to resolve owing to dense compaction. In plan view, the walls are sinuate and the over all appearance is heminiscent of a jig-zaw puzzle.

Lab notes.

COLLECTION AND PRESERVATION OF AGARICS

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University of Madras
MADRAS

Agarics can be found growing [illegible] during the ra[illegible] [illegible] on [illegible] of the [illegible] [illegible] of wood, in the [illegible], meadows, on dung [illegible] in temperate regions. [illegible] develop and decompose very quickly in [illegible] tropical climate. This [illegible] [illegible] of agarics for [illegible] study.

The equipment needed for collecting mushrooms is [illegible] [illegible] handle is essential. Baskets with lid may be useful when one gets [illegible] in sudden rain [illegible] collection. In addition to the basket one should be equipped with sheets [illegible] paper [illegible] small [illegible] plastic [illegible] knife or a hunting [illegible] pencil and a pad of paper.

[illegible] collecting agarics it is always preferable to [illegible] different stages [illegible] development of the fruit body. But this may [illegible]. If [illegible] single good mature specimen is available, it can also be collected. [illegible] take [illegible] the fruit bodies. The specimens should be carefully dug up from the substrate along with the underground part, [illegible] volva, pseudorhiza etc., with the help of the knife and it should be handled as little as possible. Then the specimens should be placed on a sheet of [illegible] paper in such a way that it can be wrapped once or twice and the ends twisted as if wrapping [illegible] sweet (Fig.1). After wrapping like this small specimens should be kept in tins or plastic boxes and the larger ones arranged in the basket. Each specimen should be given a number and notes sh[illegible] [illegible] about the [illegible] plants, smell, change of colour when bruising etc. It is always preferable to collect specimens of about 10 different species during a single day's collection rather than collecting many species which cannot be worked out in a single day. It is ideal to start collecting as early as possible [illegible] the morning and [illegible] the afternoon for studying the collections

As soon as the specimens are taken in the laboratory a spore print of the specimen should be [illegible]. For taking a spore print, the stipe of the specimen should be cut [illegible] and the [illegible] placed on a piece of white card board or white paper, gills facing downwards. Use [illegible] black paper is not [illegible] [illegible] some of the lighter colours appear white [illegible] background [illegible] white spore [illegible] can be ascertained on a white paper by viewing the paper [illegible]. Very small specimens may be placed on a clean slide with a drop of water [illegible] the cap [illegible] preferably covered by a small container. [illegible] can be [illegible]. While the spore print is getting [illegible] of the macroscopical features of the specimen. Always start [illegible] with small, [illegible] delicate specimens first. Tough leathery forms can be [illegible]. [illegible] should [illegible] all the characters which might change when the specimens [illegible] dried. [illegible] may be prepared so that one may not miss any character. A [illegible] data sheet [illegible] used in our laboratory [illegible] appended at the [illegible] of this article. It is important to note the [illegible] the

pileus and for this a standard colour chart should be used. There are many colour charts available in the market. The one which we use in our laboratory is Methuen Hand-book of Colour. It is not very expensive, easily available and many professional mycologists are now using it. A few sketches of the entire specimen and of a longitudinal section should be made in the data sheet. Care should be taken to show the exact position of the gill attachment to the stipe (Figs. 2 & 3). When the spore print is ready the colour of the print should be noted with the help of the colour chart.

After the above mentioned studies are completed the specimens should be dried properly for future reference work. Drying is the only really satisfactory way of preservation. A simple drying apparatus can be constructed by any body. The two important principles to be kept in mind while making an apparatus are a steady source of heat and good circulation of hot air around and among the fruit bodies. A set of small screens (wire mesh) fixed on to a frame and arranged one above the other will serve the purpose. A hot plate can be used as the source of heat (Fig.4). If a hot plate is not available, a couple of electric bulbs (60 or 100 W) fixed to a wooden plank would be alright. The frame with the screens and the hot plate must be surrounded by a heat proof canvas The lowest screen should be far enough from the heat source so that the wire of the screen becomes warm but not too hot to touch. The specimens should be kept directly on the screen and not over papers. When the specimens are dry they become brittle and if they are allowed to stand for 24 hours at room temperature they will absorb enough moisture to become plaint. In this condition they can be stored in cardboard boxes along with the labels. Insect damage is one of the biggest problems in tropical climate. During drying the insects present on the specimens are completely killed. But after a short time small beatles find their way to the specimens and completely destroy them. To prevent this, a few crystals of paradichlorobenzene or similar insecticides should be placed in the boxes at regular intervals. This is very important.

<u>Some books suggested for reference</u>:

Christensen, C.M. 1970. Common fleshy fungi. Burgess Publishing Company, Minn. 237 pp.

Henderson, D.M., Orton, P.D. and Watling, R. 1969. British Fungus Flora - Agarics and Boleti : Introduction. Her Majesty's Stationery Office, Edinburgh. 58 pp.

Kornerup, A. and Wanscher, J.H. 1967. Methuen Handbook of Colour. Methuen and Co., Ltd., London. 243 pp.

Lange, M. and Hora, F.B. 1967. Collins guide to Mushrooms and Toadstools. Collins, London, 257 pp.

Miller, O.K. 1973. Mushroomsof North America. E.P. Dutton and Co., Inc., New York. 360 pp.

Ramsbottom, J. 1972. Mushrooms and Toadstools. A study of the Activities of Fungi. Collins London 306 pp.

Smith, A.H. 1949. Mushrooms in their natural habitats. Hafner Press, New York. 626 pp.

Smith, A.H. 1973. The mushroom hunters'field guide revised and enlarged. The University of Michigan Press, Ann Arbor. 264 pp.

Zoberi, M.H. 1972. Tropical macrofungi. Some common species. The Macmillan Press Ltd., London. 158 pp.

SPECIMEN COPY OF DATA SHEET

Name or Number: Locality:

Habitat: Soil type, vegetation, on dung, vegetable debris etc.

Habit: - Solitary, in troops or rings

Collected by: Determined by:

Date:

Photo
or
Drawing of exterior and vertical section of fruit body:

Pileus: Diameter Shape: Colour

Surface: dry, moist, viscid, glutinous etc.

smooth, scaly, shaggy, velvety etc.

Margin: regular, wavy incurved or not

smooth, rough, furrowed striate or not

Veil, if present:
Colour
distribution at margin, whether appendiculate or dentate
filamentous or membranous

Gills: remote, free, adnate, adnexed, decurrent etc.
crowded or distant
shape
thickness width
colour when immature at maturity

features of gill edge:

Stipe: Central, eccentric or lacking shape
dimensions: length thickness
colour: when immature at maturity

consistency (whether fleshy, stringy, cartilaginous, leathery or woody)

surface characters (whether fibrillose, dry, viscid, scally or smooth)

veil, if present: Characters
volva, if present: Characters
ring if present:
whether single or double whether membranous orfilamentous
whether persistant, fugacious or mobile: whether thick or thin
whether apical, median or basal

Flesh: Colour in pileus when wet when dry

Colour in stipe when wet when dry

Colour changes if any when exposed to air:

presence or absence of milk-like or coloured fluid.

Smell: before and after cutting: relate to a common odour

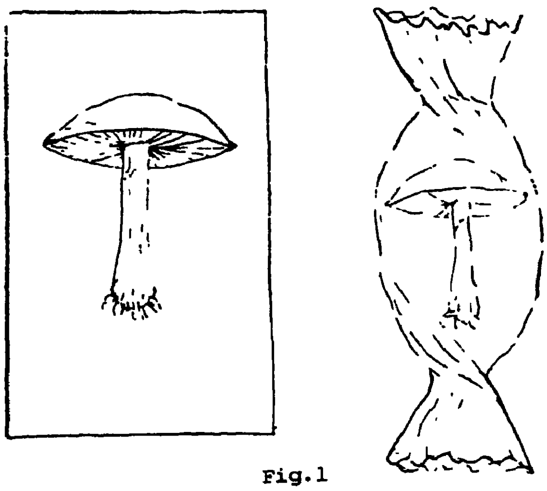


Fig.1

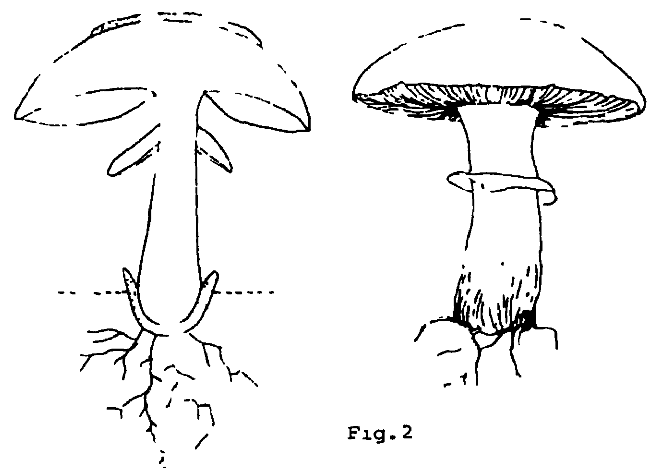


Fig.2

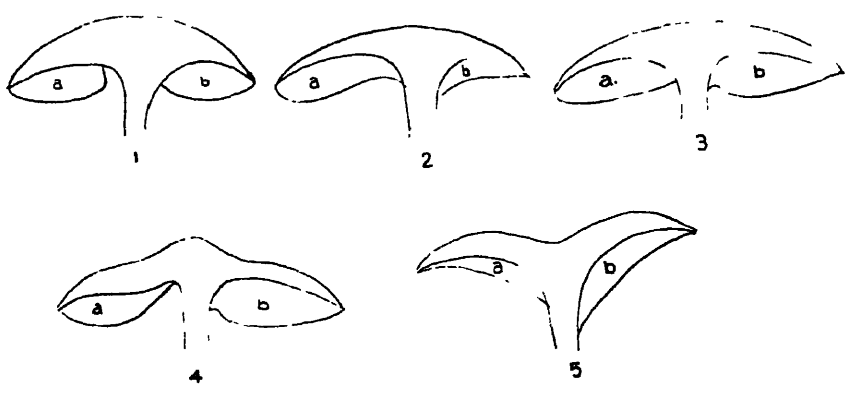


Fig.3

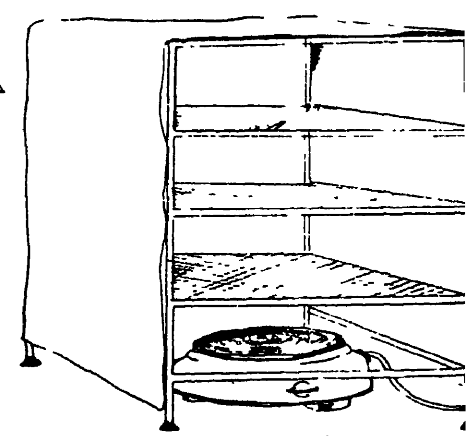


Fig.4

Fig.3. Different types of gill attachment:
1a. Remote, 1b. Free,
2a. Adnate with tooth
2b. Subdecurrent
3a. Adnate, 3b. Alucinate
4a. Adnexed, 4b. Emarginate
5a. Decurrent, 5b. Arcnate

MICROSCOPICAL STUDY OF AGARIC FRUIT-BODY

In this article methods of studying microscopic characters are described. Freshly collected fruit bodies are easy to study. If this is not available old dried materials should be first wetted for five minutes in ammonia (10% aqueous solution).

For detailed microscopic study an ordinary student's microscope with oil immersion lens and calibrated eye piece micrometer is necessary. Some of the important features to be studied are the pileus cuticle, gill trama or hymenophoral trama, stipe surface, basidia, different types of cystidia on the gills, pileus surface and stipe surface, basidiospores etc.

Malzer's reaction is very important and used as a key character especially in the white spored families. Mount a small portion of the spore print on a clean cover glass in Melzer's solution and note the amyloid reaction. The reaction is called amyloid or pseudoamyloid if positive and inamyloid if negative. The amyloid reaction is nearly black in some cases and in others it is slight pallid grayish. In the case of pseudoamyloid reaction the final colour obtained is brown to purplish brown. Inamyloid walls are yellow to nearly hyaline. Cotton blue stain can also be used as a mountant for routine microscopic studies. The spores are called cyanophilic if the spore wall adn ornamentations, if any, take the strain.

To examine the nature of the cuticle cut a 'scalp' (Fig. 1 A & B), a thin tangential section of the surface layer, mount in water or dilute ammonia and note the patterns of cells of the outer most layer around the periphery of the scalp. Care must be taken not to reverse the section when transferring to the mountant. The construction of any veil fragments will also be seen in this mount.

Remove a section of the gill along with the cap tissue and take sections as given in Fig. 1 C & D. Mount these in water containing a drop of glycerol. The outer most layer of the cap can be examined in this mount (Fig. 1 E). To spread the tissue and expose the different elements, the mount can be gently tapped with the end of a pencil with eraser attached. Cut off along line NO (Fig. 1 D) to eliminate cheilocystidia from confusing the picture.

The cortical layers are formed by a differentiated tissue forming the surface layer of the pileus. If the cortical layers is formed by septate hyphae inserted more or less perpendicularly to the surface of the organ or ascending gradually without being hymeniform it is called trichodermium. If the trichodermium is gelatinized it is called an ixotrichodermium. If the hyphae are vertical (erect) and parallel with each other, the cortex is called a trichodermial palisade, which differs from the hymeniform layer in that not necessarily every element originates and ends at the same level as the neighbouring elements of the same nature. Trichodermial palisades usually make the surface they cover velutinous or granulose or pruinose; they never show a watery, smooth, surrace. The cortical layer is called an epithelium or a cellular layer when the trichodermial palisade consists of shortened hyphal elements that tend to become sphaerocysts (isodiametric hyphae) resulting in a mass of globase to subglobose bodies arranged in a loose pseudoparenchymatous layer. If the cortical layer is formed of radially arranged or repent hyphae that are parallel to each other, it is called cutis. In some genera a dense turf like covering of normal very thin hyphae which

are often forking and branching reach the upper most layer of the cuticle. They are not always organized in such a way to be called trichodermium or ixotrichodermium. A cortical layer of this type is called 'dense' and this type occurs very frequently in the white spored families of Agaricales.

The cortical tissue itself consist of one to three layers. If there is only one it is called a cuticle (pellicle if it is viscid and peels easily). If there are more than one, the upper most layer is called epicutis. The epicutis may be a trichodermium, epitheliun, cutis or a 'dense' layer. The epicutis is followed by a inner layer called hypodermium or subcutis.

To study the basidia and other sterile elements on the hymenium cut out a wedge of tissue from the fruit body (Fig. 1 F & G) so as to have several gills attached to some cap tissue. To differentiate between pleurocystidia and cheilocystidia cut carefully along the line PQ (Fig. 1 F). Mount in water and glycerine mixture on a slide and observe under the microscope. This will show the presence or absence of cystidia on the gill face, and if present their general shape and frequency (Fig. 1 H)

The presence or absence, type, distribution, colour, shape and size of the cystidia are important diagnostic characters in all taxa. Broadly the word cystidia designates any sterile bodies that are interspersed in the hymemium or replace basidia in any part of the hymenophore or occur on one of the sterile surfaces of the carpophore resembling the hymenephoral cystidia. If the cystidia are found on the sides of the lamellae they are called pleurocystidia, if they are on the edge of the lamellae they are called cheilocysidia, if they are on the pileus surface they are called pileocystidia and if they are on the stipe they are called caulocystidia. There are also other types of cystidia based on morphology. A morphologically differentiated sterile cell arising from the same terminal subhymenial cell as the basidia and occuring on the sides of the lamellae as pleurocystidia is called a cystidiole. A cell which is more strongly differentiated than the cystidiole, thin walled and usually arising from a level lower than that of the basidia is called a leptocystidium. Hymenial extensions of the conducting system and other excretory cells in the hymemium and cystidia with chemically heterogenous contents are called pseudocystidia. There are different types of pseudocystidia as follows:-

lacteocystidia	-	with latex
gloeocystidia	-	with metachromatic oily contents
chrysocystidia	-	with amorphous bodies in the contents generally yellowish in KOH or NH_4OH mount
phaeocystidia	-	with brownish contents, weakly pseudoamyloid, containing coelospherites
macrocystidia	-	turning blue in sulfovanillin, black in sulfobenzaldehyde
coscinocystidia	-	with a sponge like body and sieve like surface
metuloids	-	thick walled (when mature) pseudocystidia with presumably excretory function in all or part of their development, often arising from oleiferous hyphae.

oleocystidia — pseudocystidia producing corpuscles in the interior which after passage through the thin partially thickened wall form a resinaceous incrustation (often pigmented and semisolid, eventually fragmented and shed from the main body).

There are also other sterile structures on the hymenium like the basidioles which are young immature basidia and setoid cystidia which are young immature basidia and setoid cystidia which are having strongly pigmented wall in the seta-like apparence and without any known excretory function.

To study the structure of the hymenophoral trama, using a low power of a microscope look down the plane of the unmodified block (Fig. 1 I) or a similar block (Fig. 1 J).

The sterile tissue of the hymenophore is called the trama or hymenophoral trama. There are 4 main types of hymenophoral trama viz., irregular, regular, bilateral and inverse. In the irregular type the hyphae are arranged completely irregularly, neither parallel to each other nor divergent. In the regular type the hyphae run in approximately or strictly axial direction (from the plane of attachment to the pileus down to the edges of the hymenophore). In the bilateral type there is a central strand which is regular but thin and an outer layer consisting of approximately parallel hyphae which are not straight or parallel with the hyphae of the thin central strand but curve outward on both sides joining the subhymenium at a point farther outwards towards the edge of the lamellae. In the inverse type the outer layer consisting of hyphae curving outwards but reaching the subhymenium further away from the gill edge rather than nearer to it as in the bilateral trama.

To study the nature of the pileus trama take out a small block of tissue as indicated in Fig. 1K. Mount and examine the structure of the outer layer of the cap and that of the flesh (homoiomerous and heteromerous) (Fig. 1 L). Remove small blocks of tissue from the stipe and mount and examine for the presence of cystidia, hair etc. (Fig. 1 M).

<u>Some of the chemicals and reagents used:</u>

Ammonia - approximately 10% aqueous solution

Sodium hydroxide - approximately 10% aqueous solution.

cotton blue -	lactic acid	-	100 gm
	Phenol	-	100 gm
	Glycerine	-	150 ml
	Water	-	50 ml

Warm mixture slightly and stir in 50 ml of a filtered 1% aqueous solution of cotton blue.

Melzer's reagent	-	Iodine	-	1.5 gm
		Pottasium iodide	-	5.0 gm
		Chloral hydrate	-	100 gm
		Water	-	100 ml

Add ingredients to the water, warm and stir thorougly. Do not boil.

Chart
Microscopic characters (add drawings)

BASIDIOSPORES:

Colour in mass　　Colour under microscope
Shape　　Size
germpore; shape, size, position
apiculus, shape, size, position
amyloidity
ornamentation

BASIDIA:

Shape, size　　Sterigmata, number and size

PILEUS TRAMA:

Type of cells

GILL TRAMA:

Type and arrangement of cells

PILEUS SURFACE:

Type of cells making up outermost layer
Presence or absence of cystidia and/or hairs

CYSTIDIA:

Presence or absence on gill face
Special features: shape, size, thickness of wall, pigmentation, if any

Presence or absence on gill edge
Special features: shape, size, thickness of wall etc.

Books and articles suggested for reference:

Smith, A H. 1966. The hyphal structure of the basidiocarp. In Ainsworth, G C and A S Sussman, Eds.
The Fungi Vol.2 : 151-177. Academic Press.

Singer, R. 1975. *The Agaricales in Modern Taxonomy* J Cramer, Lehre, 912 pp.

Watling, R. 1972. Hints on the Microscopical Examination of the agaric fruit body. *McIlvainea*, 51-58.

Explanation to Figure 1.:

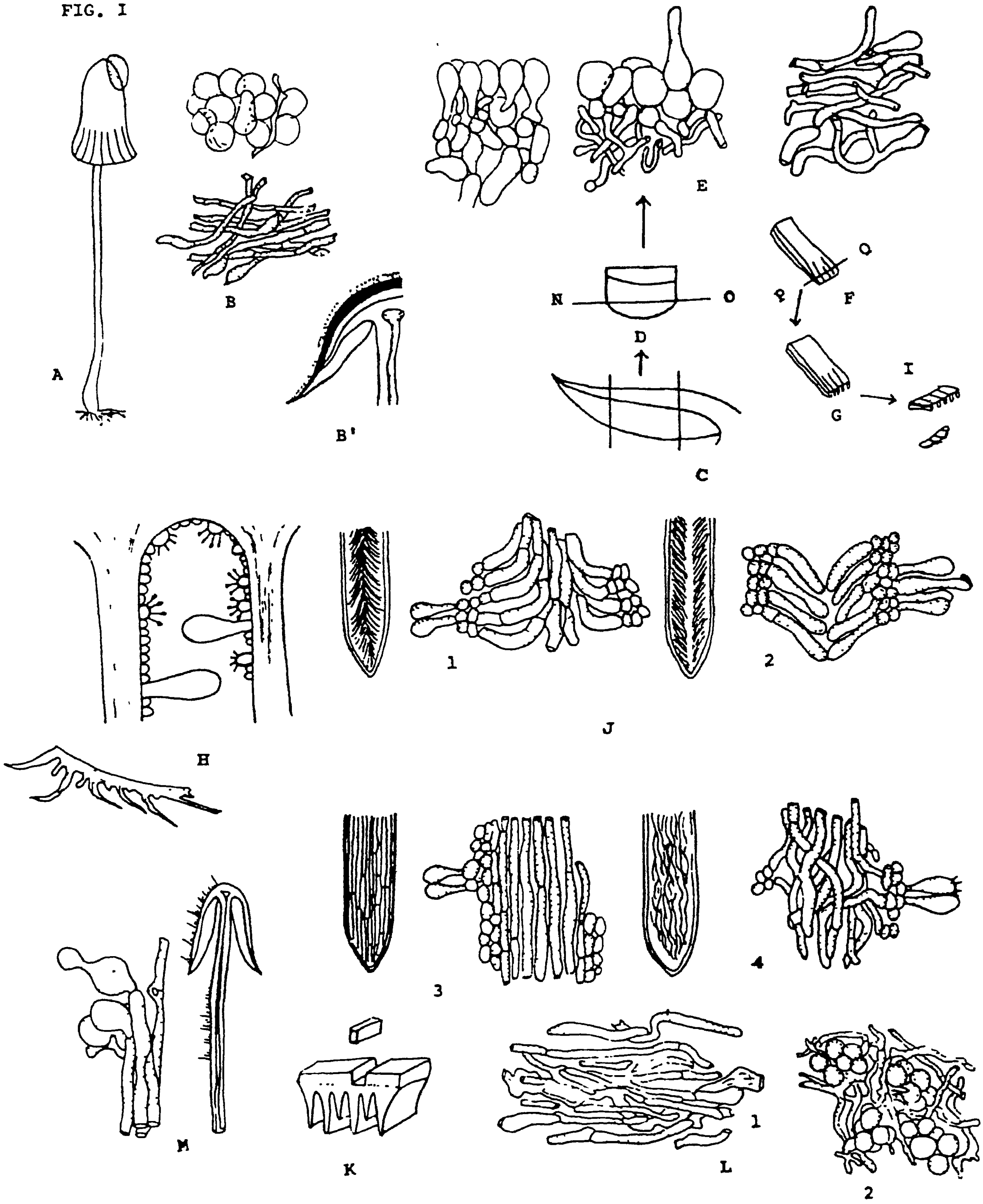
A.	Agaric fruit body with 'scalp' removed
B.	Cap 'scalp'. Top-cellular. bottom - filamentous.
B^1	Various zones of the cap tissue : a viscid layer, the outer most layer (cuticle), the underlying tissue (subcutis) and the flesh (pileus trama).
C, D. & E	Steps to observe cap tissue. Fig. E shows the L.S. of a cellular cuticle and a filamentous cuticle.
F, C. & H	Steps to observe cystidia. Fig. H shows a L.S. of gills showing cystidia.
I. & J.	Steps to observe hymenophoral trama
J1.	Divergent or bilateral
J2.	Inverse
J3.	regular
J4.	irregular
K. & L.	Steps to study flesh type
L1.	homoiomerous
L2.	heteromerous
M.L.S.	of stipe showing caulocystidia

(Figures based on Watling, 1972).

GLOSSARY

1. apiculus	-	a short, sharp but not stiff point by which the basidiospore is attached to the sterigma.
2. Coelosphaerites	-	granular internal bodies in the Phaeocystidia which are dark golden brown in Melzer's reagent under the light microscope and are electron dense, round and with a central hollow space in E.M. sections.
3. granulose	-	covered with granule like substance.
4. pallid gray	-	pale gray.
5. pruinose	-	as if finely powdered.
6. trama	-	tissue supporting the hymenium
7. velutnous	-	coated with short, fine, soft, hairy filaments arranged vertically and compactly like velvet.
8. viscid	-	sticky or slippery when moist.

FIG. I



MICROSCOPICAL STUDY OF AGARIC FRUIT-BODY

. In this article methods of studying microscopic characters are described. Freshly collected fruit bodies are easy to study. If this is not available old dried materials should be first wetted for five minutes in ammonia (10% aqueous solution).

For detailed microscopic study an ordinary student's microscope with oil immersion lens and calibrated eye piece micrometer is necessary. Some of the important features to be studied are the pileus cuticle, gill trama or hymenophoral trama, stipe surface, basidia, different types of cystidia on the gills, pileus surface and stipe surface, basidiospores etc.

Melzer's reaction is very important and used as a key character especially in the white spored families. Mount a small portion of the spore print on a clean cover glass in Melzer's solution (see p.23) and note the amyloid reaction. The reaction is called amyloid or pseudoamyloid if positive and inamyloid if negative. The amyloid reaction is nearly black in some cases and in others it is slight pallid grayish. In the case of pseudoamyloid reaction the final colour obtained is brown to purplish brown. Inamyloid walls are yellow to nearly hyaline. Cotton blue stain can also be used as a mountant for routine microscopic studies. The spores are called cyanophilic if the spore wall adn ornamentations, if any, take the stain.

To examine the nature of the cuticle cut a 'scalp' (Fig. 1 A & B), a thin tangential section of the surface layer, mount in water or dilute ammonia and note the patterns of cells of the outer most layer around the periphery of the scalp. Care must be taken not to reverse the section when transferring to the mountant. The construction of any veil fragments will also be seen in this mount.

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To study the nature of the pileus trama take out a small block of tissue as indicated in Fig. 1K . Mount and examine the structure of the outer layer of the cap and that of the flesh (homoiomerous and heteromerous) (Fig. 1 L). Remove small blocks of tissue from the stipe and mount and examine for the presence of cystidia, hair etc. (Fig. 1 M).

Some of the chemicals and reagents used:

Ammonia - approximately 10% aqueous solution.
Sodium hydroxide - approximately 10% aqueous solution.

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	Phenol	-	100 gm
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	Potassium iodide	-	5.0 gm
	Chloral hydrate	-	100 gm
	Water	-	100 ml

Add ingredients to the water, warm and stir thoroughly. Do not boil.

CHART

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BASIDIOSPORES:

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apiculus, shape, size, position
amyloidity
ornamentation

BASIDIA :

Shape, size — Sterigmata, number and size

PILEUS TRAMA:

Type of cells

GILL TRAMA :

Type and arrangement of cells

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Watling, R. 1972. Hints on the Microscopical Examination of the agaric fruit body. McIlvainea, 51-58.

Explanation to Figure 1.:

A. Agaric fruit body with 'scalp' removed.
B. Cap 'scalp'. Top - cellular. bottom - filamentous.
B'. Various zones of the cap tissue : a viscid layer, the outer most layer (cuticle), the underlying tissue (subcutis) and the flesh (pileus trama).
C, D. & E. Steps to observe cap tissue. Fig.E shows the L.S. of a cellular cuticle and a filamentous cuticle.
F, C. & H. Steps to observe cystidia. Fig. H shows a L.S. of gills showing cystidia.
I. & J. Steps to observe hymenophoral trama
- J1. Divergent or bilateral
- J2. Inverse
- J3. regular
- J4. irregular

K. & L. Steps to study flesh type
- L1. homoiomerous
- L2. heteromerous

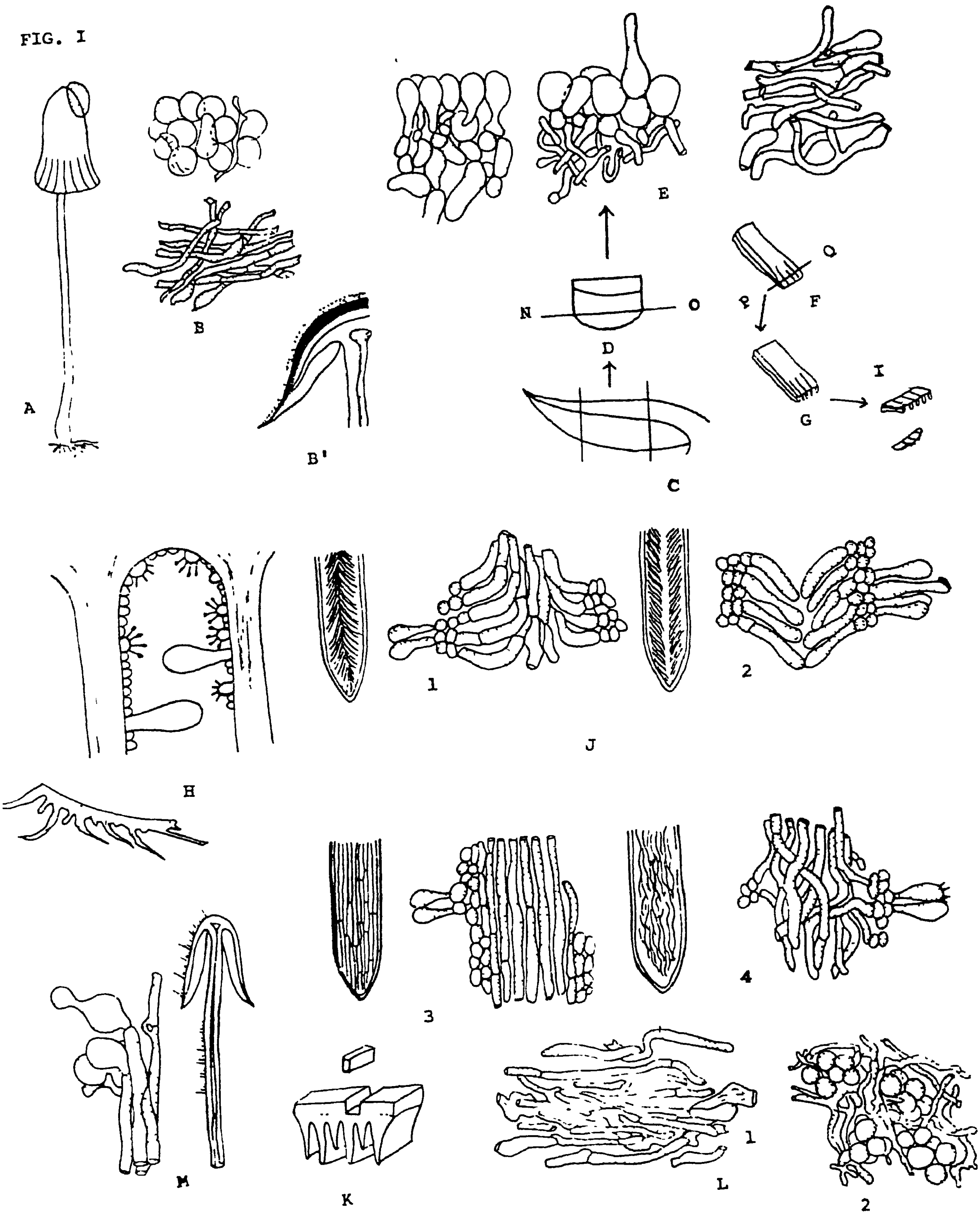
M. L.S. of stipe showing caulocystidia

(Figures based on Watling, 1972).

GLOSSARY

1. apiculus - a short, sharp but not stiff point by which the basidiospore is attached to the sterigma.
2. Coelosphaerites - granular internal bodies in the Phaeocystidia which are dark golden brown in Melzer's reagent under the light microscope and are electron dense, round and with a central hollow space in E.M. sections.
3. granulose- covered with granule like substance.
4. pallid gray - pale gray.
5. pruinose - as if finely powdered.
6. trama - tissue supporting the hymenium
7. velutnous - coated with short, fine, soft, hairy filaments arranged vertically and compactly like velvet.
8. viscid - sticky or slippery when moist

FIG. I



Eventhough Frank (1885) described the composite structures formed between some soil fungi and the fine roots of higher plants and coined the term 'mycorrhiza', Reess (1880, 1885a,b) was the first worker to identify the fungal component of the mycorrhiza. He reported the connections between the mantle of the mycorrhizas and the basidiomata of ***Elaphomyces granulatus***. In a later publication, Reess and Fisch (1887) gave detailed illustrations of the connections between the basidiomata of the fungus and the mantle of the mycorrhizas. Lewton-Brian (1901) gave further evidence of the connections between the mantle of the mycorrhizas and the basidiomata of ***Elaphomyces variegatus***. Such connections were also recorded earlier by Noack (1889). He showed the association of ***Geastrum fimbriatum*** and ***G. fornicatum*** with ***Pinus*** and ***Abies***; ***Tricholoma russula*** with ***Fagus*** and ***Pinus***; ***Lactarius piperatus*** and ***Cortinarius*** sp. with ***Fagus*** and ***Quercus***. Similarly Peyronel (1921 and 1922) also found different fungi associated with various kinds of mycorrhizas. He further showed that a particular species of fungus was not closely associated with one particular host, and that one host may form mycorrhiza with many species of fungi.

The first attempt in differentiating ectomycorrhizas on morphological basis was that of Melin (1927). His scheme was mainly based on the mycorrhizas of pines especially ***Pinus sylvestris***. He investigated the occurrence of mycorrhizal association in various types of raw humus and proposed four basic types and two sub types based on a few gross morphological characters.

Similar characterizations of mycorrhizas of ***Carya*** sp. (cultivated pecan) was carried out by Woodroof (1933). She described seven types of mycorrhizas and designated them as forms A-G. Out of these seven types, the fungal partners of three types were identified. In these three types distinct setae covering the mantle were observed. She also described and illustrated the microscopic features of the rhizomorphs attached to two types of mycorrhizas.

Dominik's (1956) classification scheme was a major development in the study of ectomycorrhizas. He incorporated detailed anatomical features in his scheme. His groupings were mainly based on distinctive characteristics of hyphal and mantle structures. Major groups were subdivided on the basis of colour of the mycelial mantle. According to macroscopic and microscopic characters of the mantle and gross

structure, the mycorrhizas were divided into 12 sub-types designated as A-L and each one of them was separated into 8 'genera' designated as a-h. He has also provided a key to identify different types of ectomycorrhizas. Dominik's (1961) system is remarkable in that it utilizes detailed macroscopic and microscopic features in defining categories. It relies especially on colour, structure and surface character of the mantle. Later Dominik (1969) published a key to ectotrophic mycorrhizas, which is today often used to designate particular mycorrhiza types collected in the field. This key includes a more detailed treatment of the different kinds of setae and cystidia covering the mantle surface. Dominik's work gave an impetus in the study of characterization of ectomycorrhizas especially the microscopic features.

Fontana (1961, 1962 and 1963) studied and described the ectomycorrhizas of several species of populus and salix growing in northern Italy. He provided line drawings of microscopic details of mantles and attached mycelia. Each mycorrhiza was designated as a numbered 'form' and classified according to Dominik's (1956) system. Fungal partners of several mycorrhizas were also identified. Later Fontana and Centrella (1967) described the ectomycorrhizas of

Castanea, Carpinus, Fagus, Pinus and ***Quercus*** formed by species of some hypogeous fungi and fungal partners of eight of them were identified. The different forms of ectomycorrhizas were also described and illustrated. They found the mycorrhizas formed by hypogeous ascomycetes characteristically have a pseudoparenchymatic mantle from which sinuous, septate hyphae sometimes emanate while those produced by hypogeous basidiomycetes have a plectenchymatic mantle from which emanate more rigid hyphae usually with clamp-connections.

Ceruti and Bussetti (1962) have studied the mycorrhizal associations of ***Boletus tomentosus, Russula grisea*** and ***Balsamia platyspora*** with ***Tilia tomentosa*** and ***Hysterangium clathroides*** with ***Tilia europaea*** and ***Tilia americana***. In this study they gave an importance to 1) the proximity and connection of carpophores with the collected mycorrhizas; 2) the structural similarities of the mycochlaena with the carpophores and 3) the similarities of the hyphae in the pure culture with those of the hyphae in the mantle.

Fassi (1965) described for the first time a form of ectomycorrhiza produced by ***Endogone lactiflua*** on ***Pinus strobus*** seedlings. He found that the mycorrhizas

produced by ***Endogone*** showed a thin fungal mantle and the mycelium extended up to endodermis penetrating through the cortical parenchyma and the intercellular hyphae formed a typical Hartig-net. The mycelial connection between the ***Endogone*** fructifications (among the pine roots in the soil) and the form of mycorrhiza is quite different from the endotrophic mycorrhiza produced by the same genus of fungus on other plants. Fassi and de Vecchi (1962) described five ectomycorrhizas of ***Pinus strobus*** nursery seedlings and provided important details of mantle structure and attached mycelia. Four of the mycorrhizal symbionts (1. ***Amphinema byssoides***, 2. ***Hebeloma hiemale***, 3. ***Ixocomus*** (***Boletus***) ***luteus***, ***I. granulatus*** and ***I. bovinus*** and 4. ***Tuber maculatum***) were identified based on proximity of sporocarps and comparison of sporocarp and mycorrhizal mycelia. In a later study, Fassi and Fontana (1966) described three forms of ectomycorrhizas of ***Pinus strobus*** in some Piedmont nurseries. The symbiotic fungal species were ***Thelephora terrestris***, ***Laccaria laccata*** and ***Hebeloma mesophaeum***. A direct mycelial connection between sporophores of these fungi and the pine roots has been demonstrated. The mycorrhizas of ***T. terrestris*** were hairy, grey and had a plectenchymatic mycochlaena; they were characterized by the presence of flexuous hairs of

various lengths and endowed with clamp-connections. The mycorrhizas of ***L. laccata*** were pubescent, white and their mycochlaena was pseudoparenchymatic in its internal part and externally plectenchymatic; the peripheral hyphae had clamp-connections and elbow-like protrusions. The mycorrhizas of ***H. mesophaeum*** were flocky, white and with plectenchymatic mycochlaena; their characters were similar to those previously found for mycorrhizas of other species of ***Hebeloma*** on pine, willow and poplar. Later Fassi and Palenzona (1969) studied the mycorrhizal associations of ***Endogone lactiflua*** with ***Pinus strobus*** and ***Pseudotsuga menziesii*** var. ***viridis***. In this study successful inoculation of seedlings of ***Pinus strobus*** and ***Pseudotsuga menziesii*** var. ***viridis*** were carried out with spores from mature sporocarps of ***Endogone lactiflua***. Typical ectotrophic mycorrhizas developed on both long and short roots. Two years after inoculation, some sporocarps were observed in pots containing the ***Pseudotsuga menziesii*** var. ***viridis***. Inoculated seedlings grew significantly taller than uninoculated, mycorrhiza-free controls.

Hacskaylo (1965) identified the ectomycorrhizas of ***Pinus virginiana*** by tracing the rhizomorphs directly from basidiomata of ***Thelephora terrestris*** to a well developed mycorrhiza. Hacskaylo and Bruchet (1972)

studied and described the mycorrhizas formed by species of ***Hebeloma***. They tested 26 species of ***Hebeloma*** from Europe for their ability to form mycorrhizas with ***Pinus virginiana***. Among the 26 species tested, 5 species did not infect short roots, 5 species penetrated only the first layer of cortical cells and the rest formed well marked Hartig-nets. They suggested that such data could be used more extensively in taxonomic studies of genera containing ectomycorrhizal fungi.

Marks (1965) studied and described seven ***Pinus radiata*** ectomycorrhizas collected from a 40-year old stand in Australia. He classified the different mycorrhizas by using the following characters: branching habit, colour, structure of mantle, microscopic details of the tannin layer, Hartig-net, cortex and endodermis. He also named six of the mycorrhizas by 'sub-type' and 'genus' according to Dominik's (1956) system.

Schramm (1966) compared the rhizomorphs of mycorrhizas with those of neighbouring basidiomata in black wastes of anthracite mining.

Luppi and Gautero (1967) described in detail the ectomycorrhizas of three species of ***Quercus*** (***Quercus petraea***, ***Q. robur*** ssp. ***robur*** and ***Q. pubescens*** ssp. ***pubescens***) with the fungal symbionts of ***Arcangeliella***,

Byssocorticium, Cortinarius, Lactarius, Russula and ***Tricholoma***. The association between ***Arcangeliella*** and ***Quercus*** was being reported for the first time. The mycorrhizas of ***Cortinarius, Tricholoma, Byssocorticium*** and ***Arcangeliella*** were generally woolly or farinose, rather slender, little branched, prosenchymatous or plectenchymatous whereas the mycorrhizas of ***Boletus, Lactarius*** and ***Russula*** were mostly fleshy and often branched with a well developed microscopic structure, pseudoparenchymatous or seldom plectenchymatous. Particularly the mycorrhizas of ***Boletus*** were often farinose (owing to the warty hyphae of the mycochlaena), those of ***Lactarius*** with a glabrous mycochlaena and also those of ***Russula*** were waxy.

Trappe (1967) described the mycorrhizas of ***Pseudotsuga menziesii*** (Douglas-fir) with species of ***Hebeloma, Suillus, Rhizopogon*** and ***Astraeus*** by pure culture synthesis. He described the mycorrhizas formed with each fungus by using gross morphology and anatomical (mantle structure, mantle hyphae, rhizomorphs and Hartig-net) characters. In this study he stressed the importance of the detailed descriptions of mycorrhizas formed by known fungi on specified hosts as a basis for mycorrhiza classification.

Rambelli (1967) described the macroscopic and the microscopic features of ten different ***Pinus radiata*** ectomycorrhizas from nursery grown seedlings. In this study the mycorrhizas were designated according to colour, form or both and the fungal symbionts were not named. Later he (Rambelli, 1970) described ten additional ***P. radiata*** ectomycorrhizas from seedlings planted on various sites by using the same characters.

In a detailed study Chilvers (1968) described eight distinctive types of ***Eucalyptus*** ectomycorrhizas from forests of New Southwales in Australia. Each mycorrhiza was fully characterized and the important details were clearly illustrated by line drawings. The two important features used for distinguishing the mycorrhizas were plan-views of lactophenol cleared mantles and structure of attached rhizomorphs. He also included the details of hyphae comprising mantles and Hartig-nets and details of those radiating into the soil. Only two fungal symbionts (***Cenococcum graniforme, Octaviana densa***) were identified by him.

Zak (1969, 1971a) made a series of studies on characterization and classification of mycorrhizas of ***Pseudotsuga menziesii*** (Douglas-fir) with distinct strains (blue and orange staining strains) of ***Poria***

terrestris and a tuberculate form by ***Rhizopogon vinicolor.*** Each mycorrhiza was fully described and illustrated. He also listed the cultural characters of the fungal symbionts. He designated the mycorrhizas by tree species plus fungus species and strain. Later he (Zak, 1971b) found that the easiest method to identify the fungal symbiont is to link the sporocarp to underlying mycorrhizas through the mycelia. This is true with those fungi which are difficult to culture. According to him the following stable features were useful in the preparation of proper keys for the identification of ectomycorrhizal types: 1) colour of mantle, 2) form, 3) presence or absence and character of surrounding mycelium, 4) presence or absence and character of attached rhizomorphs, 5) character of individual elements, 6) mantle texture, 7) chemical reagent colour reaction, 8) fluorescence in long wave ultra-violet light, 9) habitat and 10) odour and tastes.

Jayasinghe ***et al.***, (1969) carried out observations on the mycorrhizas of ***Pinus patula*** in Sri Lanka and made an attempt to culture and identify the fungus. This study revealed two structurally different mycorrhizas in the dichotomously branched rootlets. They found that the mycorrhizal fungus was a basidiomycete but could not identify it.

Marx and Bryan (1970) studied the pure culture synthesis of ectomycorrhizas by ***Thelephora terrestris*** and ***Pisolithus tinctorius*** on different conifer hosts. They used two isolates of ***T. terrestris*** and one isolate of ***P. tinctorius***. Isolates of ***T. terrestris*** produced basidiocarps and formed typical ectomycorrhizas with seedlings of following species of ***Pinus***: ***P. aristata***, ***P. banksiana***, ***P. clausa***, ***P. contorta***, ***P. echinata***, ***P. elliottii*** var. ***elliottii***, ***P. lambertiana***, ***P. nigra***, ***P. palustris***, ***P. pinaster***, ***P. ponderosa***, ***P. resinosa***, ***P. rigida***, ***P. strobus***, ***P. sylvestris***, ***P. taeda*** and ***P. virginiana*** and ***Pseudotsuga menziesii***. var. menziesii. Atypical mycorrhizas (lacking mantle) were formed on seedlings of ***Picea abies*** and ***Pinus banksiana***, ***P. densiflora*** and ***P. griffithii***. ***Pisolithus tinctorius*** formed typical mycorrhizas with seedlings of ***Pinus banksiana***, ***P. clausa***, ***P. densiflora***, ***P. echinata***, ***P. elliottii*** var. ***elliottii***, ***P. nigra***, ***P. palustris***, ***P. pinaster***, ***P. resinosa***, ***P, rigida***, ***P. strobus***, ***P. sylvestris***, ***P. taeda*** and ***P. virginiana***. They have reported that the mycorrhizas formed by ***T. terrestris*** were macroscopically and microscopically different from those formed by ***P. tinctorius*** but mycorrhizas formed by different isolates of ***T. terrestris*** were indistinguishable from each other regardless of host. These

results suggested that the fungal symbiont determined the colour and morphology of ectomycorrhizas.

In another study on ***Pinus radiata***, Mejstrik (1971) classified the ectomycorrhizas found on this host in New Zealand. He collected soil samples along with mycorrhizal roots from a 50-60 year old ***P. radiata*** stand and classified and identified eight subtypes and thirteen sub-subtypes of ectomycorrhizas. The subtype 'B' seemed to be the most common one.

Riffle (1973) studied the pure culture synthesis of ectomycorrhizas on ***Pinus ponderosa*** with species of ***Amanita***, ***Suillus*** and ***Lactarius***. In this study ***P. ponderosa*** seedlings were inoculated with pure cultures of 15 different fungal species belonging to 13 hymenomycetes and 2 gasteromycetes. Typical ectomycorrhizas were synthesized on ***P. ponderosa*** by four fungal species viz., ***Amanita muscaria***, ***A. pantherina***, ***Suillus granulatus*** and ***Lactarius deliciosus***. The remaining eleven fungi did not form mycorrhizas. He used morphological and anatomical (mantle structure, mantle hyphae, intracellular infection and Hartig-net) characters to describe the mycorrhizas formed by the four fungal species.

Thoen (1974) studied the morphology and anatomy of mycorrhizal roots on pines in exotic plantations in the Haut Shaba region (Republic of Zaire). He identified and described seven types of mycorrhizas following the system of Dominik (1969).

Froidevaux (1975) studied the mycorrhizal association of ***Pseudotsuga menziesii*** with ***Cantharellus cibarius***, ***Inocybe geophylla*** and ***Piloderma byssinum*** and characterized the mycorrhizas using Zak's (1971b) system of classification. By using pure culture synthesis method Froidevaux and Amiet (1975a,b,c) described the morphology and anatomy of the mycorrhizas of ***Pinus cembra*** with ***Suillus placidus*** and ***Suillus variegatus***; ***Pinus sylvestris*** with ***Rhizopogon rubescens*** and ***Pinus mugo*** with ***Boletus edulis*** ssp. ***edulis***. In this study the mycorrhizas were found to be ectendomycorrhiza type. They used both morphology and anatomy (mantle structure, mantle hyphae and Hartig-net structure) for describing the mycorrhizas.

Marais and Kotze (1977) described the ectomycorrhizas of ***Pinus patula*** formed by ***Boletus edulis***, ***Amanita muscaria*** and possibly by ***Tuber rapaeodorum*** and ***Lycoperdon umbrinum*** in South Africa.

Thomas and Jackson (1979) described seven types of ectomycorrhizas of nursery grown ***Picea sitchensis***. Of which four were formed by basidiomycetous fungi. Among the four fungi only two species were identified viz., ***Thelephora terrestris*** and ***Laccaria laccata***. Isolates obtained from the mycorrhizas were used to synthesize mycorrhizas under aseptic conditions. They described the mycorrhizal mantle structure, mantle hyphae and rhizomorph structures.

Chu-Chou and Grace (1983a) characterized and identified the natural mycorrhizas of ***Pseudotsuga menziesii*** (Douglas-fir) collected from nursery seedlings and forest trees. The characterization of the mycorrhizas were based on both morphological (colour of mantle, branching form, surface texture of the mantle, attached mycelia and rhizomorphs) and anatomical (mantle structure, thickness of the mantle, depth of the Hartig-net in the cortical cells) features. They characterized thirteen types of natural ***P. menziesii*** mycorrhizas and the fungal symbionts of nine of them were identified as ***Rhizopogon vinicolor***, ***Rhizopogon*** sp., ***Hebeloma crustuliniforme***, ***Amanita muscaria***, ***Endogone flammicorona***, ***Boletus*** sp., ***Laccaria laccata***, ***Thelephora terrestris*** and ***Tuber*** sp. In a later study Chu-Chou and Grace (1983b) described the mycorrhizas of radiata pine

in New Zealand. Of the 14 types of natural mycorrhizas of ***Pinus radiata*** recognized, 12 were found to be formed by ***Amanita muscaria, Endogone flammicorona, Hebeloma crustuliniforme, Inocybe lacera, Laccaria laccata, Rhizopogon luteolus, R. rubescens, Scleroderma verrucosum, Suillus luteus, Thelephora terrestris, Tuber*** sp. and ***Tricholoma pessundatum***.

A morphological classification of the ectomycorrhizas of ***Quercus*** spp. (oak) and ***Fagus sylvatica*** (beech) in the north-east of France was provided by Voiry (1981). He classified the mycorrhizas into three main groups and thirteen types based on the structure of the outer mantle and of the attached mycelia. The morphological differentiation of the three groups was correlated with the taxonomic position of the fungal species.

Acsai and Largent (1983a) described the ectomycorrhizas of selected conifers growing in sites which support dense growth of bracken fern. They have described 15 ectomycorrhizas associated with ***Abies concolor*** and 13 ectomycorrhizas with ***Pseudotsuga menziesii***. The ectomycorrhizas were described based on morphological and anatomical (rhizomorphs, mycelium, mantle structure and Hartig-net) characters. Later

Acsai and Largent (1983b) also studied the mycorrhizas of ***Arbutus menziesii*** and ***Arctostaphylos manzanita*** in northern California. In this study fifty five ectomycorrhizas were described in association with ***Arctostaphylos manzanita*** and five with ***Arbutus menziesii.*** Most of the mycorrhizas examined were found to be of arbutoid type. Some of them belonged to arbutoid-ericoid type and some others ectomycorrhizas. They used morphological and anatomical (mantle structure, attached mycelium and rhizomorphs) characters to describe each mycorrhiza. Several fungi were identified as mycorrhizal associates of ***A. manzanita*** of which the following species of ***Cortinarius*** were most common: ***Cortinarius zakii, C. fibrillosipes*** var. ***fibrillosipes.*** A species of ***Calocybe*** and ***Boletus aereus*** were also identified as mycorrhizal associates of ***A. manzanita. Cenococcum graniforme*** was found to be in mycorrhizal association with both ***A. menziesii*** and ***A. manzanita. Lyophyllum*** sp. was another fungus in association with ***Arbutus menziesii.***

In a pure culture synthesis study Palm and Stewart (1984) characterized the mycorrhizas of ***Pinus resinosa*** produced by ***Suillus americanus, S. brevipes*** (2-isolates), ***S. luteus*** and ***S. neoalbidipes,*** and ***Pinus strobus*** in association with ***S. americanus, S. brevipes***

(2-isolates), ***S. granulatus***, ***S. pictus*** and ***S. punctipes***. In all these combinations the mycorrhizas formed were similar in that all had multiple dichotomous branches, hyphae and hyphal strands with microscopic, pigmented surface incrustations surrounding the mycorrhizas and roots and in the mantle organization. But they differed in the macroscopic colour of the mantle and surrounding hyphae and hyphal strands, the amount of hyphal surface incrustations present, the organization of individual hyphae in the mantle layers, the width of the mantle, the presence of clamp-connections and the amount of radial elongation of cortical cells. The above features were used for the identification and description of the different ectomycorrhizas.

Warcup (1985) studied the ectomycorrhiza formation by ***Glomus tubiforme*** which is a white sporocarpic species with chlamydospores measuring 20-45 x 18-38 um and up to 2 um wide fine hyphae. This fungus forms ectomycorrhizas in association with a range of plants including ***Eucalyptus***, ***Pinus*** and ***Quercus***. The mycorrhizas have a thin sheath of one to a few cells thick and a Hartig-net.

In a series of papers Agerer and his co-workers have described and characterized the mycorrhizas in different plant species produced by different fungi. In

an introductory paper, Agerer (1986a) provided detailed information on the different morphological and anatomical features to be used in the characterization of ectomycorrhizas. Agerer (1986b; 1987a,b,c; 1988a,b) described the morphological and anatomical characters of ectomycorrhizas in ***Picea abies*** produced by different species of fungi (***Lactarius deterrimus***, ***L. picinus***, ***Russula ochroleuca***, ***R. xerampelina***, ***Dermocybe cinnamomea***, ***D. sanguinea***, ***Tricholoma sulfureum***, ***T. vaccinum***, ***Cortinarius obtuses***, ***C. venetus*** var. ***montanus***, ***C. hercynicus***, ***C. variecolor***, ***Thelephora terrestris*** and ***Paxillus involutus***). He has provided keys for the identification of different mycorrhizal types. The mycorrhizas were identified by tracing the hyphal connections between the basidiomata and the mycorrhizas. In his description he gave importance for the morphology and anatomy of the rhizomorphs wherever they occurred. Agerer and Weiss (1989) described the mycorrhizas formed by ***Thelephora terrestris*** on ***Picea abies***. In this study, they have compared the ectomycorrhizas isolated from the soil with that of pure culture synthesized mycorrhizas. Similarly Brand and Agerer (1986) studied the ectomycorrhizas of ***Fagus sylvatica*** (natural beech) produced by ***Lactarius subdulcis*** and ***Laccaria amethystina*** and compared this with pure culture synthesized mycorrhizas. In the sa-

work they also studied and identified the natural mycorrhizas produced ***Lactarius villereus*** by tracing hyphal connections between basidiomata and mycorrhiza. Brand (1989) described the ectomycorrhizas formed by ***Xerocomus chrysenteron*** on ***Fagus sylvatica*** by tracing its rhizomorphs to the basidioma.

Uhl (1988a) described the mycorrhizas of ***Pinus sylvestris*** produced by ***Rhizopogon luteolus***. The mycorrhizas were characterized by a coralloid habit, ochre to yellow colour, highly differentiated rhizomorphs, a two-layered mantle and Hartig-net. Later Uhl (1988b) described and compared the ectomycorrhizas formed by ***Tricholoma flavobrunneum*** in ***Betula pendula*** and ***Tricholoma auratum*** in ***Pinus sylvestris***. The ectomycorrhizas differed in the morphological characteristics of the rhizomorphs and emanating hyphae as well as in fluorescence and reactivity to brilliant-cresyl blue. Both ectomycorrhizas are white and densely stringy.

Taylor and Alexander (1989) studied the ectomycorrhiza in ***Picea sitchensis*** (sitka spruce) formed by ***Russula aeruginea*** in pure culture synthesis and compared them with the mycorrhizas produced by other species of ***Russula*** reported by earlier workers.

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INOCULUM PRODUCTION OF ECTOMYCORRIZAL FUNGI

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About 5000 fungal species are known to establish ectotrophic mycorrhizae with approximately 2000 woody plants of Gymnosperm and Angiosperm families. The plants with mycorrhizal associations are healthier, sturdier and more disease resistant than their counterparts lacking mycorrhizae. Some forest species fail to survive without mycorrhizae. Therefore, the introduction of ectomycorrhizal inoculants have facilitated plantation of tree species in areas which otherwise could not support the growth of such species.

Potential advantages of using ectomycorrhizal inocula include : (i) reduction in the amount of chemical fertilizers required due to improved uptake of nutrients by the host root-fungal hyphae network, (ii) reduction in the use of pesticides due to the decreased risk of infection in plants which have a mycorrhizal association, (iii) afforestation of areas which previously could not be used, and (iv) improvement in the quality of host plant that results in increased yields and productivity, thus ameliorating the economy of the process (Harvey *et al.* 1989, Harvey 1991).

EM fungi may be of use in land reclamation in establishing trees on landfill sites, industrial wastes such as anthracite waste, metallic mine tailings and coal spoils. The presence of these fungi on such sites appears to increase the host tolerance to drought, high temperatures, soil toxins and extremes of soil pH (Marx 1980).

The EM fungi have been introduced into nursery or forest soils by transferring natural soil containing desirable mycorrhizal fungi, incorporating sporophore material or spores and inoculating with pure cultures of vegetative mycelia. The last approach has been recommended as the most suitable method of inoculation (Trappe 1977). A major problem, however, has been the difficulty in the large scale cultivation of EM fungi.

SELECTION OF ECTOMYCORRHIZAL FUNGI

Ectomycorrhizal fungi are difficult to grow in the laboratory, and some species grow slowly. Most of them require specific growth substances such as thiamine and biotin. The fungal species are selected based on their ability to form mycorrhizae with the host, to grow in the extremes of soil

conditions, and to readily propagate in the laboratory (Harvey *et al.* 1989). *Cenococcum geophilum, Hebeloma crustuliniforme, Suillus luteus, Paxillus involutus, Laccaria laccata, Pisolithus tinctorius* are early stage fungi with a broad host range (Kendrick and Berch 1985). Since these are suitable EM fungi for inoculation purposes, submerged fermentation technology may be developed for these fungi.

INOCULUM PRODUCTION METHODS

Ectomycorrhizal fungi can be propagated by either solid state fermentation (SSF) or submerged fermentation. Different forms of inoculum available commercially are presented in Table 1. The inoculum must be inexpensive, sturdy and contain physiologically and genetically defined propagules of EM fungi.

SOLID SUBSTRATE CULTIVATION OF EM FUNGI

In Solid-substrate fermentation (SSF), the mycelium grows on the solid surface provided by the substrate itself or by inert porous material soaked in nutrient solution. Both processes of SSF have been used to produce EM fungal biomass. Moser (1958) was the first who introduced the concept of SSF for the production of ectomycorrhizal inoculum for plant seedlings. Various forms of the cultivation process are the modification of this concept. This process consists of inoculation of sterile solid substrate with mycelium grown in flask or fermenter and incubated for 3-4 months to allow mycelium to penetrate the entire matrix. After incubation, this matrix is washed with sterile distilled water to remove the residual substrate. The resultant solids are then dried to certain level of moisture content and applied as inoculum for tree seedlings. This inoculum can be stored maximally for 3-4 months at 4^{o}C.

Almost all early stage EM fungi have been tried by this method to produce inoculum. *Pisolithus tinctorius* was the first of these to be made available commercially (Marx *et al.* 1984). Sorghum grain, wheat, white millet and germinated barley have been used as substrates and solid support for propagating these fungi (Raman 1988, Takacs 1967). Vermiculite, an inert matrix, as solid surface has been used in various combinations with peat moss moistened with nutrient medium (Moser 1958, Marx *et al.* 1984). Theodorou (1967) used vermiculite, corn chaff and corn in various combinations to produce inoculum.

Solid substrate fermentation lacks precise control over various production parameters of the process. Because of this, the mycelial inoculum produced is physiologically inconsistent. Inoculum produced through SSF is bulky and difficult to maintain. Scale-up of SSF is not easy (Harvey *et al.* 1989). The process includes washing and drying at the end of fermentation, and this is not sufficient to remove residual substrate in the matrix. Further, this step makes the inoculum very viscous. Substrates

that remain in the inoculum invite saprophytes to grow over the EM fungi, leading to the loss of inoculum efficacy as well as causing wilting in plant seedlings. Long incubation time of SSF also invites contaminants during cultivation.

ADVANTAGES OF SUBMERGED CULTIVATION

There are two main advantages of submerged cultivation : (i) sophisticated fermentation equipment can be used, and it can be scaled upto volumes over 100 m^3, and (ii) under submerged conditions mycelium grows faster than in the stationary culture. Furthermore, the submerged culture of EM fungi produces higher biomass irrespective of slow or fast growing species (Sasek 1989).

SUBMERGED CULTIVATION OF EM FUNGI

EM fungi are cultivated in semisynthetic modified Melin-Norkan's medium (Marx 1969) or other media (Melin and Ram Das 1954, Palmer and Hacskaylo 1970) in the pH range 3.5 to 5.9 at 18 to 25°C under submerged conditions with high dissolved oxygen levels (Harvey 1991). Very few reports have been published on the successful cultivation of EM fungi in submerged liquid fermentation.

Intracellular (metabolic mechanisms and genetic organisation) and extracellular (nutritional and envirnomental factors) influence growth of microorganisms. Direct control of intracellular factors that influence growth is not possible. By employing sophisticated fermentation technology with nutritional control, it is now, however, possible to influence intracellular factors through controlling parameters external to the cell.

Le Tacon *et al.* (1985) cultured *Hebeloma cylindrosporum* in 175 L and 800 L fermenters for 5 days at 25°C and pH 6.0; aeration rate was 1.8 m^3/h and air pressure 1 bar. The agitation was 180 rotations min^{-1}. The liquid medium contained 10 g L^{-1} of glucose and yeast extract. Dry mycelium of 4 g L^{-1} was obtained at the end of fermentation. Sasek (1989) cultivated *Suillus luteus, Laccaria laccata, Hebeloma crustuliniforme* and *Paxillus involutus* in 20 L fermenter with 10 L of glucose-cornsteep medium. Cudlin *et al.* (1993) reported that they used 20 L fermenter with 10 L KHO medium, and 75 or 150 L production fermenters with 50 and 100 L medium agitated at 100 rpm, maintaining aeration at 0.4 VVM. After 2-3 weeks, the biomass reached 10 g dry mycelium L^{-1}. The USDA and Abbot Laboratories cultured *Pisolithus tinctorius* in modified Melin-Norkan's medium in large 20,000 L fermenters at 28-32°C for 7 days. Under optimized conditions, *L.laccata* ITCC 3334 produced 4.6 g L^{-1} of mycelium. While growing, the strain secreted polysaccharide as well as acids (Garg *et al.* 1995).

Stirred tank fermenters are often used for culturing fungi and it can be modified to suit the needs of the organism. For filamentous fungi good mixing and mass transfer conditions are essential, and the area available for attachment to surfaces within the vessel should be minimal since fungi have a tendency to adhere to surfaces. Air lift fermenters which do not have mechanical agitation system could also be useful in culturing EM fungi. Since EM fungi are aerobic and oxygen is sparingly soluble in water and culture media, the stirred tank reactor is likely to be the best type of fermenter for use to ensure an adequate supply of oxygen for their growth.

For shear-sensitive EM fungi e.g. *Pisolithus tinctorius*, *Paxillus involutus* (Boyle *et al.* 1987), the standard six blade Rushton turbine may prove to be unsuitable; a gentler agitation system such as Anne propeller may prove to be useful. All three parameters (mixing, air handling and heat transfer) are dependent on adequate bulk flows. These can be achieved by using large impeller to tank diameter ratios. Scaba agitator (four blades), Lightinin A315 (four blades) and Prochem Maxflo T (4/5/6 blades) impellers which pump broth horizontally may be very useful in culturing EM fungi in fermenters.

APPLICATION OF THE INOCULUM

The mycelium produced by submerged cultivation can be immobilized in alginate/pectin gel to produce granular inoculum for application in the nursery stock inoculation. This aspect has been recently reviewed (Cudlin *et al.* 1993). In Australia, one technological improvization in submerged cultivation for fungi has been introduced. This process was developed initially for the production of glucoamylase, later on extended to EM fungi (Kuek *et al.* 1992). In this process, the fungal biomass produced in fermentor is harvested, homogenized and entrapped in alginate matrix. Beads thus produced are incubated in growth medium at pH 2.5 till the carbohydrate is exhausted in an airlift fermentor. During this period the fungal fragments grow on the surface of beads. Beads thus produced can be used as inoculum.

CONCLUSIONS

Ectomycorrhizal inoculants provide several potential advantages to the host trees particularly on waste and degraded lands that are infertile and characterized by extreme soil conditions. The results obtained by some workers cited in this paper, clearly suggest that fermentation technology has a great potential for the bulk production of EM fungal inoculum. However, in view of very limited published information available on successful large scale submerged cultivation of EM fungi, further work in this direction is required on designing suitable fermenters and optimization of culture parameters to obtain maximum yield of mycelium.

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Table 1. Commercial availability of vegetative mycelial inoculum

Process	Inoculum form* (matrix)	Species (strain)	Commercial source
Solid-substrate fermentation			
	Vermiculite (MycoRhiz^R)	*Pisolithus tinctorius*	Abbott Laboratory USA
	Vermiculite	*P. tinctorius* *Laccaria laccata*	Sylvan spawn, Worthington PA.
	Vermiculite	*L. laccata* *Hebeloma crustuliniforme* *Paxillus involutus*	Somycel and INRA, France
	Vermiculite	*P. tinctorius*	Mycorr Tech. Inc. Pittsberg, PA
Submerged Fermentation	Alginate	*L. laccata* *P. involutus* *H. crustuliniforme* *Thelephora terrestris*	Biotal Ltd. Cordiff, UK
	Alginate	*H. crustulini forme*	Rhone-Poulec and INRA, France
	Alginate (Mycobead^R)	Eleven EM straine	Biosynthetica Pty. Ltd., Australia

INRA - Institut National de la Recherche Agronomique.

* MycoRhiz^R and Mycobead^R are trade names.

EXPERIMENT : Immobilization of the hyphal fragments of the ectomycorrhizal fungus *Laccaria laccata* in alginate.

REQUIREMENTS :

Hyphal fragments prepared by homogenization
di-Sodium alginate [4% solution]
Calcium chloride hydrated [0.1 M]
Laminar flow
Measuring cylinder [1L]
Conical flaks [2L, 1L] flasks
Beakers [2L, 1L, 500 ml]
Silicone tubes
Peristaltic pump
Buchner funnel
Micropippet tips [1000 ul]
Razor

PRINCIPLE : The immobilization of hyphal fragments in alginate matrix is due to physical entrapment of fragments in the matrix. The physical entrapment occurs when the hyphal fragment - alginate suspension is dropped into calcium chloride solution. The matrix is formed due to the replacement of sodium ions of the polymer with calcium ions. Alginate is a polymer of β-D mannuronic acid and α-L guluronic acid. Calcium ions form ionic bridges between the guluronic acid residues of the polymer. Guluronic acid content of the polymer defines the strength and porosity of matrix. Alginate polymer with low guluronic acid can be used for the production of EM fungal inoculum.

PROCEDURE : Mycelial mats of *Laccaria laccata* are obtained by growing in 250 ml flasks containing 25 ml of glucose-ammonium chloride broth* for 15 days in an incubation shaker at 30°C and centrifuging the contents. Mycelial mats are suspended in sterile distilled water and homogenised for 150 seconds at 8000 rpm.

Hundred mL each of mycelial slurry and 4% sodium alginate are mixed, and allowed to fall drop by drop into 500 ml of 0.1 M calcium chloride solution through a peristattic pump. Beads are then cured in calcium chloride solution for 1 hr.

Beads are washed with 2L of sterile distilled water and preserved in sterile distilled water at 4°C until use for inoculation of the tree seedlings.

COMPOSITION OF GLUCOSE-AMMONIUM CHLORIDE BROTH

Glucose	20 g/L
KH_2PO_4	0.5 g/L
NH_4Cl	1.0 g/L
$MgSO_4.7H_2O$	0.15 g/L
$CaCl_2.2H_2O$	0.066 g/L
NaCl	0.025 g/L
$FeSO_4.7H_2O$	10.53 mg/L
Na_2 EDTA	37.25 mg/L
H_3BO_3	2.784 mg/L
$MnSO_4.H_2O$	3.380 mg/L
$CuSO_4.5H_2O$	97 ug/L
$ZnSO_4.7H_2O$	201 ug/L
$Na_2MoO_4.2H_2O$	338.46 ug/L
Thiamine.HCl	100 ug/L
Biotin	40 ug/L
pH	6.0

Autoclaved at 15 psi for 15 minutes.

DNA Fingerprinting - RAPD and its Applications

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Principles of Genetic Fingerprinting

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Abstract

Genetic information resides in the cell nucleus of all organisms and some can be found in the organelles such as plasmids, chloroplasts and mitochondria. The size of the nuclear human genome is approximately 3 x 10^9 bp whereas the mitochondrial genome is a single circular DNA molecule of 16,569 bp in length. The amount of information a DNA molecule can potentially store is enormous.

Genomes of eukaryotes contain both single copy and repeated DNA sequences. Single copy sequences account for only a small fraction of the genome and are generally conserved within species. However, DNA polymorphism resides in the repetitive region of the genome. This is because repeated DNA sequences are not under any selection pressure. There appears to be no constraint on introducing and maintaining sequence changes in the repetitive DNA as long as there is no function. Repetitive DNA can be further subdivided, both with respect to the degree of reiteration and with respect to the location of elements of a repeat. Repetitive DNA sequences fall into three main classes:

a) Tandemly organized repeated DNA sequences.

b) Short Interspersed repeated DNA sequences (SINES)

c) Long Interspersed repeated DNA sequences (LINES)

Tandem repeats occur as satellite, minisatellite and microsatellite sequences. Satellite DNAs are dispersed over the entire genome. Minisatellites and microsatellites can be highly variable and thus form excellent tools for genetic individualization/fingerprinting. Their variability is due to arrays on a given chromosome having different repeat numbers in different individuals. Polymorphism thus created by such elements are termed variable number of tandem repeat (VNTR)

polymorphism.

Eukaryotic genomes contain short tandem repeats such as $(GATA)_n$, $(GACA)_n$, $(CAG)_n$, $(CAC)_n$, $(CT)_n$ which are dispersed throughout the genome. A number of oligonucleotide probes have been tested in DNA fingerprinting experiments. The DNA probes such as $(CAC)_5$ and its complement $(GTG)_5$ were found to be the most informative of all the probes tested in man. $(CAC)_5$ probe can differentiate each individual on the basis of RFLP signals and is thus useful for DNA fingerprinting. The overall rate of mutation in a fingerprint is substantially higher then that of an average DNA sequence. Many hypervariable loci with potentially different genetic stabilities are recorded at the same time with one probe. The ubiquitous dispersion of single repetitive DNA containing loci and their detection by oligonucleotides probes make DNA fingerprinting an attractive technique.

Human minisatellites such as 33.6 and 33.15, characterized by Jeffreys and co-workers, are highly polymorphic due to allelic variation in repeat copy number. A probe of minisatellite can provide individual specific DNA fingerprints in human, avian and even plant genomes. These probes have been useful in forensic and paternity cases. The polymorphism exhibited by minisatellites is thought to be connected to the recombinational propensity of each core sequence. These core sequences may form the basis for their duplication, amplification and promotion of unequal exchange at minisatellite loci. These sequences have also been used for studies on fingerprinting of mouse species (Jeffreys *et al.*, 1987).

Experimental details of fingerprinting using the above principles will be discussed, with special reference to their application in human systems (paternity and forensic cases)

Randomly Amplified Polymorphic DNA (RAPD) for DNA fingerprinting of plants

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Abstract

Identification and characterization of cultivars, breeding lines and hybrids is an important exercise for the breeders to ascertain the genetic purity of the product before releasing it for commercial exploitation. Morphological traits which are being used for phenotypic identification, have a limited use for the identification of closely related varieties/cultivars. With the advent of the Polymerase Chain Reaction (PCR), DNA fingerprinting becomes an attractive technique. It detects polymorphisms in the length of the amplified sequences between the annealing sites of two synthetic DNA primers. When the distance varies between two individuals then the banding pattern generated by the PCR reaction is a genetic polymorphism and can be mapped in the same way as the other genetic markers.

This procedure can amplify a targeted sequence even when it makes up less than one part in a million of the total initial sample. It is an enzymatic process that is carried out in discrete cycles of amplification, each of which can double the amount of target DNA in the sample. Thus, n cycles can produce 2^n times as much target as was present to begin with. Although PCR can detect DNA polymorphisms but it requires prior knowledge of DNA sequence. Recently, a novel technique based on the amplification of random DNA sequences by polymerase chain reaction (PCR) using arbitrary primers, 10 nucleotides in length or longer has been reported. DNA amplification with an arbitrary primer as short as 5 nucleotides can also detect DNA polymorphisms when applied to a wide variety of organisms. This approach is not only simple and rapid but also does not require the use of cloned probes and is independent of prior DNA sequence

information.

Specific amplified products segregate in Mendelian fashion and therefore they can be effectively used as (1) genetic markers to determine genetic relationships such as varietal identification, protection of breeders rights and parentage determination (2) followed in F2 segregating populations produced by crosses for the construction of linkage map (3) used to generate dendrograms of phylogenetic relationships at both intraspecific and intravarietal level. The information can be used for heterosis breeding.

Hu and Quiros used RAPD markers for the identification of broccoli and cauliflower cultivars. RAPD technique was also used for the characterization of the three diploid and amphidiploid Brassica species and for the identification of hybrid maize plants.

At our laboratory RAPD assays for fingerprinting of some Indian and exotic *B. juncea* genotypes were performed using more than thirty decamer primers. Most primers gave fingerprinting pattern that could easily distinguish between the Indian and exotic cultivars. However, only a few primers could distinguish between the closely related Indian or exotic varieties. Hence, combinations of two primers were used to generate greater polymorphism amongst the closely related genotypes. In order to investigate the relationships among the *Brassica* genotypes, pair-wise comparisons of amplification products was done and Jaccard's similarity coefficients calculated. A dendrogram was constructed using an unweighted pair group method with arithmetical averages (UPGMA). Based on this analysis the genotypes were clustered into two groups, A and B. Group A was comprised of only the exotic genotypes, whereas all the Indian genotypes and four of the exotic genotypes were clustered in group B.

Thus RAPD technique provides a practical solution for the establishment of genetic identity and partitioning of variation. This technique is being pursued by various groups worldwide.

RAPD'S in Populus

1. Poplar DNA was diluted to a working concentration of 5ng/µl
2. The DNA samples (10ng) should be aliquoted into small microfuge tubes.
3. The cocktail is made as shown on Page 8.
4. The master mix is distributed equally to each of the microfuge tubes, after a brief spin.
5. Following a momentary spin, the reaction mixture is overlaid with one drop of mineral oil.
6. The samples are placed in the thermal cycler (see Page 9 for cycling parameters).
7. Reactions are held at 4°C (soak file) following completion of the last cycle.
8. RAPD bands are resolved on a 1.5% agarose gel in 0.5 X TBE at 30 V overnight.
9. Gels are visualised by staining with ethidium bromide. **

   ** Ethidium bromide is highly carcinogenic. Handle with care.

RAPD's of *Populus deltoides*

(Final reaction volume 25μl)

Components	**Final concentration**	**Volume required (one reaction)**
10X Promega Buffer	1 X	2.5 μl
25 mM $MgCl_2$	2.5 mM	2.5 μl
Primer (15ng/μl)	15ng	1.0 μl
10 mM dNTP	0.12 mM	0.3 μl
Taq DNA polymerase (Amresco 5U/μl)	0.5 units	0.1 μl
Water		16.6 μl
Total volume		23.0 μl
DNA used (5ng/μl)	10 ng	2.0 μl

NOTE : 10 X Promega buffer is Mg free.
10 X Reaction buffer contains:
500mM KCl
100mM Tris HCl pH-9.0 (25°C)
1% Triton X-100

CYCLING PARAMETERS.

Cycle Count.	Cycling Parameters		
One	Denaturation Annealing Extension	94°C 36°C 72°C	60S 30S 60S
Thirty Five	Denaturation Annealing Extension	94°C 36°C 72°C	5S 15S 60S
One	Time delay file	72°C	7'
	*Soak File	4°C	__

* NOTE: Samples can be stored at 4°C or in the soak file also.

EXPERIMENT 1

RAPD's in *Brassica* Species

1. The protocol is identical to the one for *Populus* (Page 7-9). However the working stock is ≈ 25ng/µl and hence the cocktail to be made is shown below.

Components	Final concentration	Volume required (for one reaction)	Volume required (for 20 reactions)
10X Promega Buffer	1 X	2.5 µl	50.0 µl
25 mM $MgCl_2$	2.5 mM	2.5 µl	50.0 µl
Primer (C-15) (15ng/µl)	15ng	1.0 µl	20.0 µl
10 mM dNTP	0.12 mM	0.3 µl	6.0 µl
Taq DNA polymerase (Amresco 5U/µl)	0.5 units	0.1 µl	2.0 µl
Water		17.6 µl	352.0 µl

- 24µl of cocktail to be added to each tube
- DNA 1µl (in each tube)

1	2	3	4	5	6	7	8	9	10
B. nigra	*B. oleracea*	*B campestris*	Empty	*B. juncea* (Pusa bold)	*B. napus* (Regent)	*B napus* (excel)	*B carinata*	*B tournefortii*	*Eruca sativa 2*
11	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
Eruca sativa 3	*S. Arvensis - 8*	*Diplotaxis erucoides*	*S Arvensis*	*S. alba*	*Radish*	*Diplotaxis sirfolia*	*Erucastrum gallicum*	Marker (Kb ladder)	Control

EXPERIMENT 2

RAPD'S of *B. juncea* genotypes

Preparation of cocktail for *Brassica juncea* samples

Components	Final concentration	Volume required (for one reaction)	Volume required (for 20 reactions)
10X Promega Buffer	1 X	2.5 μl	50.0 μl
25 mM $MgCl_2$	2.5 mM	2.5 μl	50.0 μl
Primer (A07) (15ng/μl)	15ng	1.0 μl	20.0 μl
10 mM dNTP	0.12 mM	0.3 μl	6.0 μl
Taq DNA polymerase (Amresco 5U/μl)	0.5 units	0.1 μl	2.0 μl
Water		1.1 μl	22.0 μl

Dispense 17.5μl of DNA (25 ng) to each tube and 7.5μl of cocktail mix
Control has only 2μl of water

1	2	3	4	5	6	7	8	9	10
Pusa bold	Varuna	Kranti	Pusa barani	RLM514	Rohini	RC135	SRM147	B328	CSR1110
11	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
RC493	SJ11	EJ10	RC781	Pakistan collection	IC41739	Malopolaska	Vniimk	Donskaja	Marker

EXPERIMENT 3

RAPDs of *Populus deltoides* samples

Preparation of cocktail for *Populus deltoides* samples

Components	Final concentration	Volume required (for one reaction)	Volume required (for 20 reactions)
10X Promega Buffer	1 X	2.5 μl	50.0 μl
25 mM $MgCl_2$	2.5 mM	2.5 μl	50.0 μl
Primer (A07) (15ng/μl)	15ng	1.0 μl	20.0 μl
10 mM dNTP	0.12 mM	0.3 μl	6.0 μl
Taq DNA polymerase (Amresco 5U/μl)	0.5 units	0.1 μl	2.0 μl
Water		16.6 μl	332.0 μl

- Dispense 23μl of cocktail mix to each tube containing 2μl of DNA
- Control has only 2μl of water

1	2	3	4	5	6	7	8	9	10
94/91	Hybrid 2	51PD	13/86	Hybrid 3	134/86	Clone 45	55-3	PD/81	PD/70
11	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
PD154/86	Hybrid 4	47-4	PD73	22/86	P7/92	65PD	Control	Mol Wt Marker	-

DNA MINI-PREPARATION FROM *Populus*

This protocol is useful for DNA extractions from small amounts of leaf tissue and, since CsCl banding is not used, this method is quick and can easily produce 30-40 preparations in a day. All procedures are carried out tin Eppendorf tubes for speed of sample handling. An average prep. contains between 200 μg and 250 μg of DNA with quality suitable for both PCR and RFLP analysis by Southern blot hybridization.

The key component for achieving a successful prep. with high yield is careful selection and vigorous grinding of source material. We choose only the youngest, freshest, still-rolled-up leaves which on occasion have yielded as much as 1200 μg DNA from only ≈250 mg wet weight of tissue. Typical yields are ≈200 μg. The leaves are collected and firmly packed into a 1.7 ml Eppendorf tube and usually take up a volume of ≈250 μl. The collected samples can then be stored at -70°C indefinitely. If lyophilized, the leaves can be stored at room temperature, though yields from dried tissue may be lower than those fresh or frozen leaves.

1. Aliquot approximately 300 μl of glass beads (3M, #380-5005) and 600 μl of Nuclei buffer (0.3M sucrose/10mM Tris-Cl pH 7.9/1mM EDTA) into each tube.
2. Use a motorized pestle (disposable pestles by Kontes, #749520-0000 (or contact our lab for a re-usable custom-ground stainless steel pestle) to grind the leaves vigorously for 1-2 minutes or until completely homogenized. **DO NOT UNDERGRIND!** Samples can be stored on ice while awaiting further processing. If browning of ground tissue is a problem, gassing the tube with nitrogen or Co_2, after grinding will help.
3. Pellet the nuclei in a microcentrifuge, spinning for 1-5 minutes at 12-14KRPM. Discard supernatant and, with a toothpick, resuspend pellet in 750μl of **DNA extraction buffer** (100 mM Tris-Cl pH 7.9/500 mM NaCl/20 mM EDTA/1% SDS/0.1% 2-mercaptoethanol (optionally with diethyldithiocarbamic acid sodium salt freshly added to 4 mg/ml to reduce oxidation) and 100 μg of proteinase K (our yields increase at least four-fold when proteinase K is used). Incubate for 1-3 hours at 65°C.
4. Top off each tube with approximately 300 μl of TE-saturated phenol:chloroform (1:1), vortex, and centrifuge for 2 minutes.
5. Leaving all of the particulate matter behind, transfer the aqueous (top) phase to a new Eppendorf tube, fill tube with isopropanol, invert several times to mix, and centrifuge for 5 minutes.

6. Aspirate, rinse pellet with 70% ethanol, aspirate again, and allow pellet to dry.
7. Dissolve pellet in 500 μl TE (10 mM Tris-Cl pH 7.9/1mM EDTA), add 10 μg of RNase A, and incubate for 20 minutes at 37°C.
8. Extract with 250 μl of phenol: chloroform as above and after transferring the aqueous phase ($\approx$500μl) to a fresh tube, add an equal volume of 5M ammonium acetate. Precipitate the DNA as above by filling tube with isopropanol, inverting, and centrifuging.
9. Aspirate, rinse with 70% ethanol, apirate again, air dry pellet, and resuspend in 50 μl TE.
10. Quantify samples via flourometry using a 100 ng/μl genomic DNA standard.

DNA Isolation Method By Dellaporta

Procedure

1. Weigh 1 gm of leaf tissue, quick freeze in liquid nitrogen and grind to a fine powder in a 3" mortar and pestle. Transfer powder with liquid nitrogen into a 30 ml Oak Ridge tube.

   It is imperative not to let the tissue thaw once frozen until buffer is added and not to cap the tubes while nitrogen is evaporating.

2. Add 15 ml of Extraction Buffer (EB): 100 mM Tris pH 8, 50 mM EDTA pH 8, 500 mM NaCl, 10 mM mercaptoethanol.

   For maximum DNA yields, the cells are further broken by grinding the mixture at a low setting (about 3) with a Polytron (Brinkmann Instruments, Inc.). However, this step is optional.

3. Add 1.0 ml of 20% SDS, mix thoroughly by vigorous shaking, and incubate tubes at 65°C for 10 min.

4. Add 5.0 ml, 5 M potassium acetate. Shake tube vigorously and incubate 0° for 20 min.

   Most proteins and polysaccharides are removed as a complex with the insoluble potassium dodecyl sulfate precipitate.

5. Spin tubes at 25,000 x g for 20 min. Pour supernatant through a Miracloth filter (Calbiochem) into a clean 30 ml tube containing 10 ml isopropanol. Mix and incubate tubes at -20° for 30 min.

6. Pellet DNA at 20,000 x g for 15 min. Gently pour off supernatant and lightly dry pellets by inverting the tubes on paper towels for 10 min.

7. Redissolve DNA pellets with 0.7 ml of 50 mM Tris, 10 mM EDTA, pH 8. Transfer the solution to an Eppendorf tube. Spin the tubes in a microfuge for 10 min to remove insoluble debris.

8. Transfer the supernatant to a new Eppendorf tube and add 75 ul 3 M sodium acetate and 500 ul isopropanol. Mix well and pellet the clot of DNA for 30 sec in a microfuge. Wash pellet with 80% ethanol, dry, and redissolve in 100 ul 10 mM Tris, 1 mM EDTA, pH 8. Precipitation from 0.3 M sodium acetate using relatively small amounts of isopropanol (about 0.6 volumes) has been reported to separate high molecular weight DNA from polysaccharides (Marmur, 1961). The sodium acetate also yields a tight fibrous precipitate that is easily washed and dried. The DNA will dissolve readily if allowed to rehydrate at 4°C for 1 hr followed by light vortexing.

Minipreps can be stored for several months without evidence of degradation and can be cut with a variety of restriction enzymes and ligated without further purification. The expected DNA yield from leaf tissue of most species is 50-100μg per gram tissue and is greater than 50kb.

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MANAGEMENT OF DISEASES IN FOREST NURSERIES

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INTRODUCTION

Healthy and quality seed and disease free seedlings are necessary for raising healthy and vigorous stands of any forest tree species. Tree seed and nursery seedlings may suffer damage due to pathogenic organisms (fungi, bacteria, viruses, mycoplasma like bodies etc.), insect pests, abiotic agencies (low and high temperatures, low and high soil moisture, air pollution) and deficiency of micro-or macro-nutrients. Damage due to fungi exceeds the damage caused by all other agencies put together.

Seedlings in nurseries are more prone to attack by pathogenic organisms than those growing under natural conditions as nursery grown seedlings have more tender tissues, grow in artificial ecosystems and are concentrated in a limited space. Nurseries thus present an ideal situation for epidemic spread of diseases which may sometimes destroy the entire seedling stock grown during a season. Protection of nursery plants from the attack of pathogenic organisms should, therefore, form an integral part of any nursery management programme. In the present publication, control of nursery diseases is discussed under four heads namely seed care, care of cuttings, nursery soil care and seedling care.

SEED CARE

Seeds are subjected to attack by fungi under three varying environmental conditions i.e. on trees during ripening processes, during storage and during germination in soil. Fungal infection on seeds may destroy seeds, may affect their viability or may cause diseases in seedlings before their emergence from soil (pre- emergence damping off) or after their emergence (post emergence damping off). Use of healthy and mature seeds is, therefore, necessary for raising healthy seedlings. Following measures are recommended for ensuring

health of seeds

SEED COLLECTION AND GRADING

Seeds should be collected from healthy and vigorously growing trees. Collection of seed from forest floor should be avoided except in case of large, heavy and bulky seeds. Before storage, the seeds should be graded and dwarfed, deformed, fungal infected, insect infested and immature seeds should be separated from seed lots and rejected. Seeds are often mixed with chaff or dirt which may consist of leaf bits, small bits of branches, cone and needle bits (in conifers) and other extraneous matter. Such chaff may be colonized by pathogenic organisms and this may help in transporting disease organisms from seed stands to the nursery site. Seeds after collection from forest areas should, therefore, be completely freed from all extraneous matter before storage or before sowing in nurseries.

SEED STORAGE

Seed drying : Fungi cause heavy losses in seeds during storage either by complete decay of seeds or affecting their viability thereby reducing germination percentage. Seeds after collection should be shade dried to bring down moisture content to 10-12% based on overdry weight. Cones and seeds which can withstand sun heat can be dried in sun. Cones etc can also be dried under solar heated Kilns.

Some seeds, however, loose their viability if the moisture content is lowered as in case of seeds of *Azadirachta indica, Syzygium cumini*, oaks, *Cinnamomum* spp., *Parsea gratissima.* Such seeds need to be stored under fresh conditions only. Some seeds like *Cedrus deodara, Juniperus macrocarpa, Michelia champaca* require stratification at low temperature under moist conditions. Such seeds cannot also be dried.

Treatment with fungicides : It has been found that treatment of seeds with seed dressing fungicides before storage not only checks spread of fungi from infected to healthy seeds within a seed lot but in some cases eradicates the fungal infections present on seed surfaces (Sujan Singh and Suresh Chandra 1989). Three fungicides namely captan, thiram and

organomercurial fungicides containing 1% mercury have been found as effective seed dressing fungicides. Organomercurial fungicides available in Indian markets include aretan, parasan, agrosan, cerasan among others. The active ingredients in these fungicides are phenyl mercury acetate or ethyl mercury chloride or their mixture. Seeds should be thoroughly mixed with the fungicide dust preferably in a mechanical mixer and the superfluous fungicide may then be separated from seeds using iron mesh sieves. It has been found that 2-4 g of fungicide is sufficient for treatment of 1 kg of seeds.

Seed containers: Seeds should be stored in air tight containers or in muslin cloth after drying and treatment with fungicides. Seeds should not be stored in polyethylene bags as in such containers, moisture tends to build up creating conditions favourable for fungal attack. Seed should be stored at 4-5°C temperature.

SEED SOWING

Sowing depth : In nursery depth of seeds sowing plays an important role. As a thumbrule seeds should be covered with soil layer twice the thickness of seeds. In very small seeds as in eucalypts, a thin layer of sand or soil sand mixture should be spread over the seeds.

Treatment with fungicides : In addition to the above treatment seeds can also be treated with fungicides immediately before sowing in nurseries. Two types of treatments may be given to seeds before sowing. These are seed dip treatment and seed pelletting.

In seeds where systemic infections by fungi are suspected as in seeds of leguminous tree species, dip treatment in fungicide solution helps in eradicating such systemic infection. Seeds of *Leucaena leucocephala* are often found with systemic infection of *Fusarium semitectum* which not only decays the seeds before germination but may attack seedlings causing stem canker and dieback. It has been found that internal seed infection by *F. semitectum* can be completely eradicated by giving dip treatment to seeds in 0.1 per cent water solution of emisan (methoxy ethyl mercury chloride with 6% mercury) for 15 minutes and then keeping the seeds in water for 24 hours before sowing. (Sujan Singh *et al.* 1983). Such treatment was found to increase germination percentage of treated seeds also.

Some seeds are destroyed by birds, rats, squirrels and other animals in seed bed nurseries. Pelletting of seeds with fungicides protects the seed from such type of damage. In seeds of hardwood tree species, pelletting with organomercurial fungicides, captan and thiram have been found effective in controlling pre-emergence damping off diseases. A slurry is prepared by mixing fungicides in water (50 g in case of organomercurial fungicides and 140 g incase of thiram and captan in one litre of water). 20 to 22 kg of seeds can be treated with one litre of slurry thus prepared (Nene and Thapliyal 1979). A sticker can be added to the slurry for proper sticking of fungicides to seed surfaces. Pelletting of seeds is done 24 hours before sowing.

CARE OF CUTTINGS

Some tree species like poplars are reproduced through stem cuttings. As the cuttings are collected from trees these may carry diseases from plantation to nurseries in active stage. Some diseases continue to progress on sprouted cuttings and subsequently may kill the plant. Dip treatment of cuttings in 0.1% emisan for 15 minutes helps not only to eradicate any fungal infection present on cuttings but protects the cuttings from soil borne pathogens also when these cuttings are grown in nurseries.

NURSERY SOIL CARE

Nursery practices differ with different tree species. In some species like *Eucalyptus*, seeds are first sown in seed bed nurseries and 3-4 week old seedlings are then pricked in polythene bag soils. In some species seeds are directly sown in polythene bag soil. In still others, seeds are sown in nursery beds and seedlings are then excavated with ball of earth for field plantings. In teak, seeds are sown in nursery beds and root-shoot cuttings are obtained from these seedlings for planting in the field. In all such cases, methods for treatment of nursery soil are same for control of damping off diseases.

SELECTION OF NURSERY SITE

Many nursery troubles can be avoided if nursery is selected at a suitable site. Sites with heavy, wet and poorly drained soils should be avoided for establishing nurseries. A deep, fresh light sandy loam soil meets most of the nursery requirements. As far as possible, nursery should be located at a safe distance from any diseased plantation. Too much frosty pockets and areas prone to high winds should also be avoided. Where high winds are regular features as in plains of Punjab, Haryana and Uttar Pradesh, suitable wind breaks may be provided around nurseries. Location of nursery should be as much near the planting areas as may be possible. The pH of nursery soil as well as that of water used in the nursery should not be too high or too low. For hardwoods, it should be nearly neutral and for conifers between 5.6 to 6.5

TREATMENT OF NURSERY SOIL

Damping off diseases in nurseries can be effectively controlled by treatment of nursery soil with chemicals which are used for bringing soil pH to desired level or by disinfecting soil.

Soil acidification

Alkaline soils with pH more than 7 favour epidemic development of damping off diseases in nurseries. In such soils, pH should be brought down to desired level before raising tree seedlings. Experiments with treatment of soil with sulphuric acid (250 ml of commercial sulphuric acid diluted in 4 litres of water applied 13 days before sowing to app. 1m x 1 m x 15 cm nursery bed) have revealed that this treatment not only lowered soil pH but effectively controlled damping off diseases in pine nurseries though Reddy and Misra (1970) observed that with this treatment germination percentage and number of surviving seedlings did not increase over control possibly due to phytotoxicity. Elsewhere flowers of sulphur or ground rock sulphur @ 100 g, concentrated sulphuric acid @ 160 c.c. diluted in 10 litres of water, aluminium sulphate @ 200 g and ammonium sulphate equivalent to 100 g of sulphur were effective to lower soil pH of 1m x 1m x 15 cm nursery bed soil by one unit (Benzion 1965).

Soil treatment with formalin

Treatment of nursery soil with formalin is effective mainly against pre-emergence damping off diseases though post-emergence damping off diseases are also kept under control in formalin treated soils. In pine nurseries, 250 cc of commercial formalin diluted in 4 litres of water and added to 3ft x 3ft x 6 inches nursery bed soil not only controlled damping off diseases but significantly increased survival percentage over control beds (Reddy and Misra 1970). However, failures in control of damping off diseases by formalin treatment are also often reported. Such failures may be due to inadequate amount of formalin going into nursery bed soil. In order to make the formalin treatment effective, 300 cc of formalin should be uniformally mixed with 1 m x 1 m x 15 cm (raised from ground) nursery bed soil. This can be achieved by using that amount of water for diluting the formalin which should be just sufficient to thoroughly wet the nursery bed soil without run off in the allays. This amount of water can be calculated for a particular nursery by wetting a test bed with pure water. Formalin treatment should be done 8-10 days before sowing the seeds.

Soil treatment with protectant fungicides

Captan, thiram, zineb, blitox and emisan are the main protectant fungicides which have been successfully used in various nurseries to control pre-and post-emergence damping off diseases. Each fungicide is used @ 25- 50 g in 1 m x 1 m x 15 cm nursery bed soil. The dosage is decided on the basis of the texture of nursery soil i.e. the heavier the texture the more quantity of fungicide would be needed. The fungicide is first thoroughly mixed with small quantity of soil and this soil fungicide mix is thoroughly raked up with the nursery soil. Seed can be sown in the treated soil immediately after this treatment.

SEEDLING CARE

COMMON SEEDLING DISEASES

Seedling diseases commonly occurring in forest nurseries can be discussed under three groups i.e. collar rots, root rots and diseases of aerial parts of seedlings.

Collar rots

Collar rots are most common when the seedlings are in juvenile or succulent state. Symptoms of collar rots manifest as discoloured areas on the stem at ground level. Affected areas become brown, depressed and with the death of cambium the seedling is girdled at ground level. The affected seedling at this stage usually gets toppled over. Collar rot may be caused by pathogenic organisms or by chemical or insolation injury. In collar rots of pathogenic origin the leison extends well beyond the girdled region both in the stem and in the root below the soil. In collar rots caused by heat or chemical injury, the tissues both above and below the girdled region remain healthy without any leisons. Collar rots of pathogenic origin are mainly caused by species of *Pythium* and *Phytophthora* though *Fusarium* spp. also sometimes cause collar rots. The collar rots due to pathogens become epidemic in water logged soils with poor drainage.

Root rots

Root rots many be caused by species of *Fusarium, Rhizoctonia, Phytophthora, Pythium, Cylindrocladium, Sclerotium* among others. In root rots caused by *Fusarium* spp. the affected root becomes swollen and the bark can easily be taken off like a bangle from the root. In root-rot caused by *Rhizoctonia* spp. or *Sclerotium* spp. hard, round sclerotia are always found on affected roots or in soil surrounding diseased roots. In Cylindrocladium root rots tufts of white conidiophores will appear clothing the root when diseased roots after thoroughly washing are kept under moist conditions. Roots attacked by *Phytophthora* spp and *Pythium* spp. become decayed and absorb moisture to the extent that water can be squeezed from the infected roots.

Diseases of aerial parts

A variety of pathogens attacks aerial parts of the seedlings. Species of *Cylindrocladium* can attack leaves, twigs and main stem. Eucalypts are highly susceptible to *Cylindrocladium* diseases. *Leucaena leucocephala* seedlings suffer from stem and twig cankers caused by *Fusarium semitectium*. The leaves of seedlings may be attacked by rusts, mildews and leaf spot/leaf blight/leaf curl/shot hole/anthracnose pathogens. Attack of rusts on leaves, stem and twigs of seedlings is characterized by presence of orange yellow pustules on affected regions.

Important rust pathogens in trees species raised under social forestry programmes include *Olivea tectonae* attacking leaves of *Tectona grandis, Maravalia achroa* on leaves and juvenile twigs and *Uredo sissoo* on the leaves of *Dalbergia sissoo, Ravenelia* spp. on leaves, pods and twigs of *Albizzia* spp., leaves of *Acacia* spp. *Pongamia* spp., *Dasturella divina, Tunicopsora bagchii* and *Puccinia* spp. on bamboos among others. Powdery mildews are characterized by profuse white powdery growth almost covering entire leaf surface. Many powdery mildews caused by species of *Phyllactinia* and *Uncinula* occur on nursery seedlings of *Tectona grandis, Dalbergia sissoo, Morus alba,* poplars, *Salix* spp. among others. Leaf spots are characterized by localized killing of small leaf areas, leaf curl by curling of affected leaves and leaf blight by dying of whole leaf. A variety of pathogens causes these symptoms. Common among these are species of *Colletotrichum, Alternaria, Helminthosporium. Fusarium, Phyllosticta, Myrothecium* among others. In addition to fungal diseases as discussed above, seedlings in nurseries may also suffer from diseases caused by bacteria, viruses, mycoplasmas, high or low temperature, excessive or deficient soil moisture, deficiencies due to macro-or micro- nutrients and air pollution. Viral diseases are characterized by leaf yellowing, mottling, curling or vein clearing.

CONTROL OF SEEDLING DISEASES

Seedling diseases in forest nurseries can be controlled by sound nursery practices and by use of fungicides and these are discussed below:

Potting mixture

Use of wrong potting mixture predisposes polythene bag seedlings to attack of disease pathogens. A mixture of soil, sand and farm yard manure in adequate proportion to give a light textured, loamy potting mixture with a pH between 6 and 7 is ideal for healthy growth of tree seedlings. Care should,however, be taken that the soil is not collected from diseased plantations, sand is of neutral pH and farm yard manure is sufficiently decomposed. While adding fertilizers to potting mixture care should be taken that level of phosphorus is kept higher than that of nitrogen as excess of nitrogen predisposes seedlings to attack of pathogenic fungi.

Sanitation

Maintenance of adequate sanitation in a nursery goes a long way in checking epidemic spread of diseases. Dead seedlings should be immediately removed and destroyed. Diseased seedlings should be kept separately from healthy seedlings and sprayed with fungicides. Dried leaves, twigs and roots of previous year's seedlings are often colonized by pathogenic fungi. Such material should not get mixed with potting mixture but instead should be carefully collected and buried underneath the soil.

Care during pricking

If the seedlings are damaged during pricking, these may subsequently die or become severely kinked (Sujan Singh and Khan 1983). Donald (1971.) estimated that 60 per cent of *Pinus radiata* in South African nurseries had a kinked root system attributed to poor transplanting. Older seedlings with long tap root and profuse laterals are more prone to pricking damage than young seedlings with tap root devoid of laterals. Pricking should preferably be done when laterals had just started developing. At this stage, the planting shock would be minimum and the seedlings are likely to produce healthy root system. Pricking damage can altogether be eliminated if seeds are directly sown in polythene bags. Failure of germination of seeds will, however, involve increase in expenditure on the cost of polythene bag and their filling. To overcome this, Kaushik and Singh (1972) have recommended sowing of presprouted seeds in raising chir pine plants.

Tending

Many of the serious troubles can be avoided if seedlings are properly tended after pricking in polythene bags. Too much watering which can create water logged conditions or inadequate watering wherein the soil in lower portions of polythene bags may remain dry predispose seedlings to attack of pathogenic fungi. Collar rot pathogens are favoured by too much watering while development of *Fusarium* wilt is favoured by inadequate watering. During hot and dry summer evaporation of water from surface soil results in accumulation of salts which may cause injury at collar region. Covering the surface soil with leaf mulch or with small pebbles may help in checking evaporation of water from surface soil. Leaves used as mulch have the inherent danger of introducing dangerous pathogens in the nursery. It is,

therefore, advisable to use mulch of leaves of broad leaved species for conifer seedlings and mulch of grasses for nursery seedlings of broad leaved species.

Seedlings may be affected adversely by summer heat. The affected seedlings become stunted and may die. Heated surface soil may cause girdling of the stem at soil level (collar rot) resulting in death of seedlings. Partial shading of polythene bag seedlings during hot summer may be necessary to protect the seedlings from heat injury. Watering during morning in summer months also helps to protect the plants from heat injury. Girdling of stem by heat injury may be checked by covering surface soil with leaf mulch or pebbles. Too heavy shading, however, favours development of pathogenic fungi and should be avoided.

USE OF FUNGICIDES

Use of fungicides in polythene bag seedlings becomes necessary if the disease breaks out in epidemic form. Collar rots caused by species of *Pythium* and *Phytophthora* are controlled by drenching of polythene bag soil with 0.3 per cent water suspension of any copper oxychloride fungicide containing 50 per cent active ingredient. Drenching may be repeated after three weeks if the disease persists. Fusarium root rot diseases are controlled by drenching polythene bag soil with 0.2 per cent water suspension of thiram or captan or 0.1 per cent solution of emisan (methoxy ethyl mercury chloride with 6 per cent mercury). Rhizoctonia root rot is controlled by drenching the soil with 0.2 per cent solution of brassicol (Pentachloro nitrobenzene). Leaf and twig blight of *Eucalyptus* seedlings caused by species of *Cylindrocladium* is controlled by spraying the affected plants with 0.2 percent solution of bavistin. The plants should be sprayed upto the point of dripping.

Foliage diseases are caused by a large number of fungal pathogens. No single fungicide can be prescribed for control of these pathogens. However, rust pathogens and powdery mildews are amenable to control by sulphur based fungicides. *Maravalia achoroa*, the leaf and twig rust of *Dalbergia sissoo* is controlled by spraying of plants with 0.2 per cent water suspension of dithane M-45 or 0.3 percent water suspension of cosan, a micronised sulphur fungicide. Most of the foliage pathogens are controlled by spray of leaves with 0.2 per cent suspension of any of the copper oxychloride fungicides or dithane M-45 or 0.2 percent water solution of bavistin.

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Introduction of Ectomycorrhiza in Forest Nurseries

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Introduction

In natural ecosystems, most of the trees have mycorrhizal associations without which the trees cannot survive. In temperate forests ectomycorrhizal associations dominate while most of the trees in tropical forests have endomycorrhizal associations. Ectomycorrhizae occur on feeder roots of most valuable tree species belonging to families Abeitaceae among Gymnosperms and Betulaceae, Fagaceae, Salicaceae, Juglandaceae, Myrtaceae, Caesalpinaceae, Dipterocarpaceae, Euphorbiaceae and Casuarinaceae among Angiosperms. Ectomycorrhizae benefit their tree hosts in a variety of ways. They increase absorbing surface of infected roots through inducing profuse branching. The extra-matrical hyphal growth of ectomycorrhizal fungi can explore larger soil areas for acquisition of nutrients from the soil. The hyphae of ectomycorrhizal fungi are able to mobilize nutrients from the substrates where such nutrients are in unavailable form for absorption by tree roots. The fungal hyphae assimilate nutrients from such substrates through their active metabolic process and these assimilated metabolites are then transported to tree roots. Ectomycorrhizae can store major nutrients like phosphorus, nitrogen, potassium and calcium in their mantles. Ectomycorrhizae also protect plant roots from soil-borne pathogens either by acting as barriers against penetration of roots by root pathogens or by producing antibiotics which restrict the growth of root pathogens. Ectomycorrhizal associations in tree roots also help the trees to tolerate drought, high or low soil pH, high concentration of heavy metals in soil and high or low temperatures. In order to drive maximum benefits from ectomycorrhizae, it is necessary that nursery raised seedlings have optimum level of mycorrhization on their roots for which introduction of mycorrhizae at nursery stage should form an integral part of nursery management practices.

Importance of nursery soil inoculation with mycorrhiza

Afforestation sites are usually devoid of any ectomycorrhizal fungal inoculum. Tree species specially exotics fail to establish on this site due to absence of ectomycorrhizae on their roots. The success is however, achieved when ectomycorrhizal fungi are introduced in such sites. For twenty years, repeated attempts to raise pine plantations in Puerto Rico failed due to lack of ectomycorrhizae. The pine seedlings used to grow to 5-30 cm height, then became chlorotic and died. Transfer of ectomycorrhizal soil from successful pine plantations in the main land to Puerto Rico nurseries helped in establishment of ectomycorrhizae in Pine seedlings resulting in successful establishment of pine plantations in this island. In three years time inoculated plants of slash pine grew healthy and reached a height 2-2.5 meters while uninoculated plants could reach to 5-30 cm height, became chlorotic and died. Similarly, in

South Africa, success in raising Pine Plantations was achieved only when ectomycorrhizal inoculum from Netherlands was introduced in South African nurseries. Soil from South African pine nurseries was then used as soil inoculum in Kenya and this resulted in large scale successful pine plantations in that country. In India, a large number of exotic pines was introduced in dome areas of Orissa and Andhr a Pradesh and in Nilgiries. In dome areas, pines were raised after clearfelling sal forests. Ectomycorrhizal fungi from sal successfully established on pine roots. Mycorrhizal inoculum was also taken from North Indian pine plantations and introduced in pine nurseries in dome areas. Successful establishment of ectomycorrhizae in pine nurseries resulted in successful establishment of pine plantations in dome areas.

In reforestation sites with natural mycorrhizal propagules, non-mycorrhizal nursery raised seedlings of pines fail to grow healthy for 1-2 years after planting in the field. Normal growth occurs only after 1-2 years after successful ectomycorrhizal establishment.

It is clear from the above that both in afforestation and reforestation sites ectomycorrhizal introduction at nursery stage is necessary to raise healthy plantations.

Methods of introduction of ectomycorrhizae in nurseries

Several methods are being used for introduction of ectomycorrhizal fungi in nurseries of pines and other ectomycorrhizal plants. These are briefly discussed below.

Soil Inoculum

Introduction of ectomycorrhizae in forest nurseries by the use of soil inoculum is being widely practised all over the world. Soil inoculum contains mycorrhizal roots, spores, chlamydospores, hyphae, hyphal strands, rhizomorphs and other propagules of mycorrhizal fungi. In India inoculation of potting mixture with soil inoculum is a common practice in all pine nurseries. The procedure for inoculation of nurseries with soil inoculum is discussed under three heads namely collection of soil inoculum, its storage before use and application in nursery soil.

(i) Collection of soil inoculum : It should be ensured in the first instance that soil inoculum is collected from tree plantations of the same species which is intended to be raised in nurseries. Extensive researches have revealed that mycorrhizal fungi establishing ectomycorrhizae in nursery seedlings are subsequently replaced by other fungi in field plantings when the plantations reach 6-10 years age. Also ectomycorrhizal fungi occurring in old plantations are not efficient mycorrhiza former in nursery seedlings. Soil inoculum should therefore be collected from young plantations only. It should also be ensured that soil inoculum is collected from healthy, disease free plantations only so that the risk of migration of serious disease pathogens from plantations to the nursery is eliminated.

For collection of soil inoculum, all the decayed leaves, branches and other debris should be removed upto app. 2 m radius from the base of a healthy tree from where the soil inoculum is to be collected. The soil then should be collected from upto 10 cm. depth as ectomycorrhizae are active within 10 cm. of surface soil only. It should also be ensured that the soil collected as mycorrhizal inoculum contains sufficient number of mycorrhizal short roots.

(ii) Storage : The whole operation of collection and introduction of soil inoculum in nurseries should be completed in minimum possible time as the delay may affect the viability of propagules of mycorrhizal fungi. In any case, the soil inoculum should not be stored for more than 3-4 weeks. Before storage, it should be ensured that the soil is neither too wet nor too dry. Wet soil would encourage the growth of mould fungi which may replace or even parasitize ectomycorrhizal fungi. Dry soil may desiccate and kill the propagules of ectomycorrhizal fungi. Soil moisture of 30-35 per cent based on oven dry weight is most suitable for maintaining the viability of soil inoculum. Wet soil inoculum should therefore be dried in shade before storage. Soil inoculum may be stored in gunny bags or piled up in a room on a cemented floor and covered by gunny cloth. No plastic sheets should be used for storage of soil inoculum as such a material would conserve soil moisture and encourage mould growth.

(iii) Application of soil inoculum : Before application, the soil inoculum should be thoroughly mixed so that the propagules of mycorrhizal fungi get evenly distributed in the soil. Application of soil inoculum in nursery is done by the following methods.

Mixing with soil : Soil inoculum is mixed with potting mixture in 1:10 ratio (one part soil inoculum in 10 parts of potting mixture). The potting mixture thus inoculated is filled in polyethylene bags and seedlings are pricked into the inoculated soil.

If the seedlings are raised in nursery beds, a 1 cm thick layer of soil inoculum is evenly spread over nursery bed and then the nursery bed soil is thoroughly raked upto 10 cm. depth to spread the inoculum evenly in the soil.

Inoculation of pricking holes : Soil inoculum in this method is applied in pricking holes at the time of pricking the seedlings in polybags. A 2.5 cm. diameter hole is made in the centre of polybag soil and the seedling is held in upright position in the centre of this hole. The soil inoculum is then filled in the pricking hole and the pricked seedling is compacted by the surrounding soil.

In pine seedlings it has been found that mycorrhizal development on roots of seedlings takes place 3-4 months after pricking the seedlings in polybags. It may therefore be better if inoculation of polybag pine seedlings is done two months after pricking. For late inoculations, 4-6 holes of 1 cm. diameter may be made around the seedling and filled with soil inoculum.

Inoculation with spores

Spores of ectomycorrhizal fungi are being commonly used for introduction of mycorrhizal fungi in *Eucalyptus* and pine nurseries. The spores are easy to collect, easy to store, cheap to transport and can be taken to long distances. Spore inoculum is commonly used in case of ectomycorrhizal fungi belonging to class Gasteromycetes, the members of which form fruit bodies in the form of puff balls and truffles.. Such fruit bodies are full of powdery mass of spores when they are mature. These spore masses while still enclosed in the peridiums of the fruit bodies can be stored at 4-6°c for 6-12 months without losing their viability.

Following methods are being used for spore inoculation of nursery soils.

(i) **Spore Soil mix** : In this method, approximately 2 mg of spore powder (containing app. 1×10^{12} spores) is thoroughly mixed with 1 kg of sterilized or fumigated soil and this soil spore mix is used as inoculum

For inoculation of nursery beds or potting mixture, one kg of soil-spore mix is evenly spread over one sq.m. of nursery bed soil or over one sq.m. of potting mixture evenly spread out in 1m x 1 m x 15 cm wooden frames. The soil is then thoroughly raked up (upto 10 cm depth in nursery bed soil) to uniformally distribute the spore inoculum in the soil.

Soil-spore mix can also be filled in planting holes while pricking the seedlings in polybags or in 3-4 holes made around 2-3 month old poly bag seedlings as described earlier under soil inoculum.

(ii) **Seed encapsulation with spores** : Spores of Gasteromycetous fungi can also be used for encapsulation of seeds of their host plants. A slurry is made by suspending spores in water and a sticker is added to the slurry. The seeds are then dipped in the slurry and kept overnight before sowing in nursery beds. The spores get evenly distributed in soil during watering of seed beds.

(iii) Spore pellets : In Philippines, at National Institute of Biotechnology and Applied Microbiology, Laguna, Prof. R.E. Dela Cruz and his colleagues have produced mass inoculum of ectomycorrhizal fungi in the form of tablets which have been given the trade name as 'MYCOGROE' tablets. These tablets consist of basidiospores of ectomycorrhizal fungi combined with soil as carrier and pallitized in a tabletting machine. These tablets have been successfully tested by the above workers in Philippines in nursery seedlings of *Eucalyptus camaldulensis, E.deglupta, Pinus caribaea* and *P. kesiya*. The beneficial effect of nursery inoculation of the above species with *MYCOGROE* tablets continued after out planting in the field.

Inoculation with sporophores

In some ectomycorrhizal fungi belonging to Agaricales, collection of sufficient quantity

of basidiospores for nursery soil inoculation may be difficult to obtain. In such fungi, the entire sporophore is used for inoculation. Freshly emerging agarics are collected, crushed and mixed with ten times its volume of sterilized or fumigated soil. The soil-sporophore mix is then used for inoculation of nursery soil or nursery seedlings as described earlier in soil and spore inoculum procedures. Unlike spores, sporephores cannot be stored for long and best results are obtained with freshly collected sporophores only.

Use of mother seedlings

In some African nurseries and in Indonesia, mycorrhized seedlings are used for mycorrhization of nurseries. Introduction of mycorrhizae in *Pinus merkusii* in Indonesia is mainly done through use of mycorrhizal seedlings, also called mother seedlings. This method of inoculation is also referred to as Indonesian Method. In this method, fully mycorrhized seedlings left out in successful nursery bed or obtained from the forest sites are planted evenly scattered at 1-2 m spacing in the nursery bed and the pine seedlings are pricked around these mother seedlings. Ectomycorrhizal infection slowly spreads from mother seedlings to the surrounding transplants.

Pure culture inoculum

Use of pure culture inoculum of ectomycorrhizal fungi for inoculation of forest nurseries have many advantages over other forms of inocula. The risk of introduction of dangerous pathogens into the nurseries is eliminated by this method. In soil inoculum, there is always a danger of introducing serious disease pathogens either through fungal propagules present in soil or through infected leaves, needle bits, twigs which may be inadvertently collected with soil inoculum. Production of sporophores of mycorrhizal fungi is also governed by vagaries of nature. In some years, abundant fruit bodies may develop while during years experiencing less rainfall, fruit bodies may not develop in abundance for collection for nursery inoculation. Also most efficient fungi suitable for different hosts under different edapho climatic conditions can be selected and introduced in forest nurseries by this method. Introduction of ectomycorrhzal fungi with proved efficiency can ultimately result in replacement of less efficient ectomycorrhizal fungal symbionts already esixting in nursery soil and planting site by more efficient and easily cultivable ectomycorrhizal fungi. A large number of ectomycorrhizal fungi have been successfully cultivated on synthetic media though some recalcitrant species such as some *Russula* spp. have defied all attempts to bring them in pure culture. Most of the successfully cultured fungi are slow growing and difficult to produce on mass scale for use for nursery inoculation. For mass production an ectomycorrhizal fungus should be easily culturable, should have fast growth and should have a capacity to compete well with other ectomycorrhizal fungi. It should also be an efficient mycorrhiza former, have a wide distribution and should have the characteristics of forming ectomycorrhizal associations with wide spectrum of hosts including both Gymnosperms and Angiosperms. A good number of ectomycorrhizal fungi with all or most of the above characteristics have been identified. These include *Pisolithus tictorius, Rhizopogon luteolus, Scleroderma* spp,. *Laccaria laccata, Hebeloma crustuliniforme, Paxillus involutus, Suillus*

luteus, S.granulatus and *S.bovinus*

Two types of techniques are being used for the production of pure culture inoculum of ectomycorrhizal fungi. These are solid medium culture and liquid medium culture.

(i) Solid medium inoculum : In this method, the ectomycorrhizal fungus is first grown in liquid medium and then transferred to flasks containing sterilized peat and or vermiculite moistened with liquid medium. Many liquid media are known to grow ectomycorrhizal fungi, of which the following two have been found most suitable.

· *Hagem's medium* : Glucose 5g; malt extract 5g; 0.5g each of KH_2PO_4, $MgSO_4$, $7H_2O$, NH_4Cl; 10 drops of 1% solution of ferric chloride, distilled water 1 litre (pH 4.6-4.8).

Modified Melin and Norkran's medium (MMN) : $CaCl_2$ 0.05g; NaCl 0.025g; KH_2PO_4 0.5g; $(NH_4)_2HPO_4$ 0.25g; $MgSo_4$, $7H_2O$ 0.15g; $FeCl_3$ (1% solution) 1.2 cc; glucose 10g; malt extract 3g; thiamine hydrochloride 0.1 mg; distilled water, 1000 CC (pH5.5-5.7)

Before use, the inoculum is leached under several washings of tap water to remove unutilized sugars and other nutrients. The medium from flasks after being fully colonized by ectomycorrhizal fungus is mixed with sterilized soil (1:25 ratio) and kept moist till it is used for inoculating nursery soil. 1-1.5 kg of soil-medium mix is sufficient to inoculate $1m^2$ of nursery soil or 1 mx 1 m x 15 cm potting mixture spread out in a wooden frame.

United states Department of Agriculture, Forest service in collaboration with Abbott Laboratories have produced mass inoculum of *Pisolithus tinctorius* for use at commercial scale in a solid-state fermenter containing MMN nutrient medium. Vermiculite-peat moss is used as a solid substrate. Later, two other firms namely Sylvan Spawn Laboratory, USA and Somycel, France who are specialized in mushroom spawn production started producing mass inoculum of ectomycorrhizal fungi on a commercial scale. The ectomycorrhizal fungi produced on mass scale by these firms were *Laccaria laccata, Hebeloma crustuliniforme, Paxillus involutus* and *Pisolithus tinctorius.* The ectomycorrhizal inoculum was produced by these firms on peat-vermiculite mixture in specially designed polyethylene bags with a breather strip allowing oxygenation. These bags can stand sterilization. The nutrient solutions used by the firms are such that the peat vermiculite mixture does not require to be leached before use.

(ii) Hyphal culture in liquid media : In this method mycelial cultures of ectomycorrhizal fungi are produced by liquid industrial fermentation procedures. The hyphae produced in the fermenters are then entrapped in polymeric gels, made into pellets and then used for inoculation of nurseries. LeTacon and his collegues at INRA- Centre de Nancy, Laboratoire de Micro boilogie, Fobestiere, France have used the pellets thus made successfully in forest nurseries. The mycelium produced in fermenter is first leached to remove excess nutrients, then suspended in a solution of sodium alginate (10g/l) containing clay or milled peat Small pellets of entrapped mycelium are then obtained by replacing the sodium ions by calcium ions. The inoculum can be stored for at least 10 months at 4°C. The pellets are more easy to manage in nursery and have been found to be more effective, than peat moss-

vermiculite inoculum by LeTacor and his colleagues.

In philippines, Prof. R.E. Dela Cruz and his oolleagues have produced mycelial beads, trade named as 'MYCORRHIZAL BEADS' by entrapping mycelia of *Pisolithus tinctorius* grown in liquid fomenters in calcium alginate beads. The MYCORRHIZAL BEADS were tested by them on seedlings of *Eucalyptus camaldulensis, Pinus caribaea* and *P.kesiya* with very encouraging results.

Kropacek and his colleagues. (In Agriculture Ecosystem and Environment 28 ! 263-269, 1989) have produced mass inoculum of ectomycorrhizal fungi in the form of granules. The mycelia of ectomycorrhizal fungi *Laccaria laccata* and *Suillus luteus* were grown in submersion cultures in fermenters on corn steep medium. After rinsing with distilled water, the mycelium is homogenized to obtain fragments about 1-3 mm in size. An aqueous solution of sodium alginate (2%) was mixed with perlite in 1:1 or 1:1.5 (V/V according to the size of the perlite particles) and one part of this mycelium homogenate was added to four parts of the perlite/alginate mixture. Granules were prepared by pressing this paste through a 4 mm mesh sieve into a column of 5 per cent calcium chloride. After 30 minutes, granulated inoculum was rinsed with distilled water and stored at 5°C. Granulated inoculum was found very effective in *Picea abies* and *Pinus sylvestris* seedlings

Entrapped mycelieal inoculum have many advantages over solid medium inoculum: By this technique, the mycelium is produced in 1-2 weeks while in solid techniques, several months are needed to produce the inoculum. The dosage in entrapped mycelial technique is $2g/m^2$ while in solid medium technique, 0.5-1.5l of inoculum is required to inoculate $1m^2$ of nursery bed.

ECTOTROPHIC MYCORRHIZA

Ectotrophic mycorrhiza occurs naturally on feeder roots of most valuable tree species belonging to family Abietaceae among Gymnosperms and families Betulaceae, Fagaceae, Salicaceae, Juglandaceae, Myrtaceae, Caesalpinaceae, Dipterocarpaceae, Euphorbiaceae and Casuarinaceae among Angiosperms. The fungi forming ectomycorrhizal associations do not penetrate the cells of the root tissues, hence the prefix ecto-is applied to this type of mycorrhiza.

Morphology

Root system in ectomycorrhizal plants is heterozoic comprising of long roots of unlimited growth and short roots of limited growth. The short roots are short lived. Root hairs are absent in ectomycorrhizal roots. Ectomycorrhizal fungi are attracted towards fine roots of the plant by the presence of carbohydrates and complex root metabolites present on the surface of the roots. Penetration is through enzymes produced by the invading hyphae. The infected root ceases to grow further, becomes swollen, exhibits various tints of colours depending on the colour of the mycelia of the invading fungus and becomes profusely branched. The profuse branching of the root after colonization by the ectomycorrhizal fungus is believed to be due to production of auxins by the mycorrhizal fungus (Slankis 1951). Meristematic tip and the vascular cylinder are not invaded by the ectomycorrhizal fungus. In cortical cells also, the fungus is restricted to the intercellular spaces only. Both the fungal and the root cells retain their vital characteristics without showing any pathological symptoms. The branching of ectomycorrhizal roots is characteristic of the tree species as well as the ectomycorrhizal fungus. The branching may be simple or monopodial as in spruce, fir and sal; coralloid as in deodar; forked and often showing repeated dichotomy as in pines or emulate a pyramid like structure as in eucalypts (Bakshi 1974). The fungal symbiont may vary between the short roots of the same tree species or within the fork of the same short root or in rare cases within the same branch of the fork.

Structure

Mycorrhizal roots are completely covered by a fungal sheath or mantle which completely replaces the epidermis. From the mantle, the fungal hyphae spread into the surrounding soil. These radiating hyphae as well as loose hyphae in the mantle possess all the characteristics of the fungal symbiont, on the basis of which the identity of the fungal symbiont may be tentatively determined. These radiating hyphae may be thin or thick walled, broad or narrow, simple septate or with clamps, with or without cystidia, setae like structures, chlamydospores, intercalary or apical swellings. These hyphae may also get organized into sclerotia, hyphal strands or rhizomorphs which help the fungal symbiont to explore greater soil area as well as to tide over the unfavourable soil conditions.

The mantle is usually compact with a few to several layers of organized fungal elements. Hyphae in the mantle may be loose forming a loose hyphal type mantle as in case of ectomycorrhizae formed by *Scleroderma geaster* in slash pine and chir pine. In such mantles, the individual hyphae retain their identity. In some mantles, the hyphae may be more organized but the mantles are compact and not loose hyphal. Such mantles are formed by *Scleroderma verrucosum* in eucalypts and *Hysterangium* sp., in spruce and deodar. In more compact mantles, the identity of the fungal elements is completely lost. Such mantles may be meliaceous as in sal where fungal symbionts are species of *Scleroderma*. In very compact mantles, they may be cellular or pseudoparenchymatous as in case of ectomycorrhizae formed by *Giastrum fimbriatum* in fir; *Amanita verna* in sal; *Astraeas hygrometricus* in sal and chirpine; *Cortinarius* sp. in spruce and fir and *Pulvero boletus shoreae* in sal. Mantles formed by *Cenococcum geophilum* are composed of pallisade like layers of fungal elements.

The hyphae from the mantle also penetrate the intercellular spaces of cortical tissues and form a net like structure called Hartig net. In short root infections, the mantle is invariably accompanied by the Hartig net. In long root infections, however, the mantle may be absent and only Hartig net may be produced.

Fungal symbionts

Most of the fungal symbionts forming ectomycorrhizae belong to the order Agaricales of the class Hymenomycetes and orders Hymenogastrales and Lycoperdales of the class Gasteromycetes under subdivision Basidiomycotina of the fungi. *Cenococcum geophilum*, a common black fungal symbiont forming black ectomycorrhizae belongs to Ascomycotina. *Glomus tubiforme* belonging to Zygomycetes has also been found to form ectomycorrhizae with many plant species.

Ectomycorrhizal fungi are usually identified from the sporophores developed by these fungi on the infected short roots. Such sporophores would appear as if they are growing on soil but on careful excavation of these sporophores would reveal their connection with the underground mycorrhizal roots. A sporophore may be tentatively identified as that of a fungus which had formed the ectomycorrhiza with the tree roots if such underground connection with mycorrhizal root is established. Further confirmation is however necessary by making pure culture from the sporophore of the candidate fungal symbiont and conduct synthesis of ectomycorrhiza by artificial inoculation of the roots.

A large number of ectomycorrhizal fungal symbionts have been successfully cultured on synthetic media and syntheses of ectomycorrhizae with host roots have been obtained to confirm their ectomycorrhizal nature. The ectomycorrhizal fungal symbionts belong to the genera *Amanita, Boletus, Cantharellus, Clitopilus, Clitocybe, Cortinarius, Calvatia, Cenococcum, Corticium, Elaphomyces, Entoloma, Geastrum, Hebeloma, Laccaria, Lactarius, Leccinum, Lepiota, Lycoperdon, Pisolithus, Paxillus, Rhizopogon, Russula, Scleroderma, Suillus, Thelephora, Tricholoma, Xerocomus* among many others. Most of the fungi belonging to the above genera are able to develop ectomycorrhizae with a broad spectrum of tree species. Species of *Scleroderma* and *Pisolithus* form ectomycorrhizae with tree species among

both Gymnosperms and Angiosperms. Also, a **single tree may have a variety of fungal** symbionts in its ectomycorrhizal associations.

Functions of ectomycorrhiza

Ectomycorrhiza forms a very important component of forest ecosystem. Mycorrhizal roots are more effective in uptake of inorganic and organic nutrients from the soil in comparison to non-mycorrhizal roots due to increased absorbing surface provided by extensive proliferation of ectomycorrhizal short roots, and by fungal hyphae radiating from the mantle into the soil. In some substrates, the nutrients are in unavailable form for the uptake of plant roots. Mycorrhizal fungi are able to mobilize nutrients from such substrates by their active metabolic process and then translocate such nutrients to host plants as assimilated metabolites. Ectomycorrhizae store nitrogen, phosphorus, potassium and calcium in their mantles and slowly release these elements for utilization of plant roots. Ectomycorrhizae are most effective in soils which are deficient in phosphorus, nitrogen and/or potassium.

Ectomycorrhizae are known to protect plant roots from soil-borne root pathogens. Such protection may be afforded by mantles serving as mechanical barriers against penetration of root pathogens or due to production of antibiotics that can restrict the growth of some pathogens. It was first shown by Marx (1966) that ectotrophic mycorrhizal fungi in pine root mycorrhizae acted as barriers to invasion of pine tissues by *Phytophthora cinnamomi*. Since then, a lot of work has been published on control of soil borne root pathogens by ectomycorrhizal associations.

Ectomycorrhiza also benefits woody plants in imparting tolerance against drought, high or low soil pH, high or low soil temperature and heavy metal concentrations in the soils.

Nutrition of Ectomycorrhizal fungi

Most of the ectomycorrhizal fungi have been found by different workers to be capable of utilizing a variety of carbon sources. Different carbon sources which have been tested include, glucose, fructose, xylose, maltose, lactose, sucrose, pectin, inulin, starch, cellobiose, glycerol, manitol among others. Some fungi may however have preference for certain carbon sources over the others. Cellulose is not utilized. A pine root ectomycorrhzal fungus *Boletus subtomentosus* behaves as litter decomposing fungus as it is capable of forming polyphenol oxydase abundantly. In general glucose has been found as the best carbon source for a majority of ectomycorrhizal fungi.

Ectomycorrhizal fungi are able to utilize varied types of nitrogen sources also. Nitrogen sources tested by various workers are ammonium chloride, ammonium nitrate, ammonium sulphate, ammonium tartarate, potassium nitrate, arginine, glutamic acid, glycine, casein hydrolysate, urea, asparagin, tryptophane (Bakshi, 1974; Subashini, Natarajan 1995).

Among vitamins, thiamine, riboflavin, pyridoxine hydrochloride, Biotin, inositol,

nicotinamide, calcium pentothenate and folic acid were tested by Bakshi and his colleagues with *Corticium bicolor, Scleroderma bovista* and *Cenococcum graniforme* (Bakshi 1974). All the above fungi have been shown deficient in thiamine and to a lesser extent riboflavin. Other vitamins did not have much effect on the growth of the above fungi when compared with control.

Most of the ectomycorrhizal fungi grow best at pH range from 5-6 though some may grow well upto pH7 also.

Management of ectomycorrhizae in nurseries

Inorganic fertilizers are generally applied to forest nurseries to achieve a healthy seedling stock. Excess of inorganic fertilizers suppress ectomycorrhizal development. When such healthy seedlings are planted in the field especially on infertile soils, they soon die or show poor growth due to absence of ectomycorrhizae. Ectomycorrhizae are most effective in soils moderately deficient in phosphorus, nitrogen and potassium. As early as in 1937, Hatch found increases of 86 percent of nitrogen, 234 per cent of phosphorus and 75 per cent of potassium in ectomycorrhizal pine seedlings over non-mycorrhizal plants. Use of inorganic fertilizers in forest nurseries should be avoided unless there is a major deficiency of any of the macro-nutrients. Dinesh Kumar, Sujan Sing, Thapar, Misra (1968) found that optimum development of ectomycorrhiza in chir pine nurseries occurs when nutrients are present in the soil at half the normal level (normal level being 130 ppm of both nitrogen and phosphorus; 39 ppm potassium). Ectomycorrhizal development was moderate at one fourth normal level but was poor at normal and higher levels.

Effect of pesticides

Use of fungicides is a normal practice in forest nurseries to control damping off diseases. Fungicides are reported to exert depressing effect or may not have any effect or may have stimulating effect on development of ectomycorrhizae. Fungicides like fermate, captan and benomyl are reported to stimulate ectomycorrhizal fungi while fungicides like dithane M-45, triadimefon and its Isomer triadimenol are reported to suppress ectomycorrhizal development. It is, therefore, necessary that the fungicides which are compatible with ectomycorrhizal fungi should only be used in forest nurseries. There are reports that most of the insecticides at lower or prescribed doses are compatible with ectomycorrhizal development.

Effect of shading

In forest nurseries, shading of nursery plants during hot summer days becomes necessary to protect them from insolation injury. Shading suppresses ectomycorrhizal development. Only partial shading should, therefore, be practised so that photosynthetic activities of the plants are not affected. Shading for 3 hours does not affect development of

ectomycorrhizae in chirpine seedlings while 6 hour's shading suppresses mycorrhizae (Bakshi 1974).

Introduction of ectomycorrhiza in nurseries

In natural ecosystems, mycorrhizae are indispensable components of plant roots and the very survival of trees depends on this mutualistic association (Hacskaylo 1976). In afforestation programmes especially where exotic tree species are being introduced, success of tree plantations depends on successful establishment of ectomycorrhizae in tree roots. In reforestation sites also though mycorrhizal inoculum is usually present in the site, yet non-mycorrhizal nursery seedlings fail to develop ectomycorrhizae during first one to two years after transplanting. It is therefore necessary that in all forest nurseries introduction of ectomycorrhiza should form an integral part of nursery management practice.

Commonest method which is practised in forest nursires for introduction of ectomycorrhiza is through use of infected soil collected from healthy young plantation of the same tree species which is being raised in nurseries. The mycorrhizal soil is mixed with potting mixture in 1:10 ratio or filled in planting holes at the time of pricking the seedlings into polybags.

In case of Gasteromycetous ectomycorrhizal fungi, spores produced in puff ball or truffle type fruit bodies are commonly used for inoculation of tree seedlings in nurseries. Spores are mixed with sterilized or fumigated soil and this soil-spore mix is then used for inoculation of forest nurseries. Encapsulation of seeds with spores is also done for ectomycorrhizal introductions in forest nerseries. In agarics where sufficient spores collection may be a cumbersome process, crushed fruit-bodies of ectomycorrhizal fungi are also used for nursery inoculation.

In some nurseries of South Africa and in Indonesia, seedlings with adequate ectomycorrhiza are collected from the forest or raised in the nursery and then transplanted in nursery beds at scattered places. Nursery seedlings are then pricked into nursery bed soil around these ectomycorrhizal or mother seedlings. The ectomycorrhizal fungi slowly spread in soil and infect the seedlings surrounding the mother plants. Introduction of ectomycorrhizae in *Pinus merkusii* in Indonesia is mainly practised through use of mother plants.

Use of pure culture of ectomycorrhizal fungi is by far the best method for introduction of ectomycorrhiza in forest nurseries. This method eliminates the risk of introduction of desease pathogens which can establish in nurseries through soil inoculum. A large number of ectomycorrhizal fungi have been cultured on synthetic media and some more promising ones have been produced on mass scale for use in nurseries.

Mass pure culture inoculum production is being produced on solid media and with liquid medium technology. In solid medium method, ectomycorrhizal fungi are first grown in liquid medium and then transferred to flasks containing sterilized vermiculite or peat-vermiculite mixture moistened with the liquid nutrient medium. This inoculum needs leaching

in running tap water to remove unutilized nutrients.

In liquid medium technology, liquid indestrial fermentation procedures are employed. The hyphae produced in such liquid medium are then entrapped in polymeric gels. The mycelium produced is first leached under tap water to remove unutilized nutrients and then resuspended in a solution of sodium alginate (10g/1 litre) containing clay or milled peat. Small pellets of entrapped mycelia are then obtained by replacing sodium ions by calcium ions.

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